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THE GLUCOSE METABOLISM OF *PLASMODIUM GALLINACEUM*, AND THE ACTION OF ANTIMALARIAL AGENTS

BY

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Studies on the metabolism of the erythrocytic forms of plasmodia were initiated by Christophers and Fulton (1938), who showed that monkey red cells infected with *P. knowlesi* consumed oxygen independently of glucose, though added glucose was utilized ; the oxygen uptake of the parasitized cells was completely inhibited by cyanide. Maier and Coggeshall (1941) showed that parasitized red cells were dependent to some extent on glucose, and that mannose, fructose, and glycerol would replace glucose as a metabolite. During the war years, a lot of research on the metabolism of the malaria parasite has been done by various groups of workers in America. This work has been comprehensively reviewed in *Federation Proceedings* (vol. 5, No. 3). It was reported that, under aerobic conditions, red cells infected with malaria parasites could metabolize lactate, pyruvate, succinate, fumarate, and amino-acids, besides glucose, and that glucose was converted to lactic acid by phosphorylation, as in muscle (Evans, Ceithaml, Speck, and Moulder, 1945 ; Bovarnick, Lindsay, and Hellerman, 1946a, b ; McKee, Ormsbee, Aufinsen, Geiman, and Ball, 1946). Speck and Evans (1945a) showed that cell-free extracts of plasmodia would phosphorylate glucose similarly to yeast extracts.

Regarding the action of antimalarials, Silverman, Ceithaml, Taliaferro, and Evans (1944) reported that quinine and mepacrine (atebrin) inhibited the respiration of plasmodia, though the inhibition was delayed until the third or fourth hour of incubation with the drug. Speck and Evans (1945b) reported that quinine and mepacrine inhibited the phosphorylation of glucose by cell-free extracts of plasmodia and of normal red cells, and that the degree of inhibition corresponded to the amount of hexokinase activity present. Investigating isolated enzyme systems, these workers showed that quinine and mepacrine inhibited yeast hexokinase, but not 3-phosphoglyceraldehyde dehydrogenase. Lactic dehydrogenase from plasmodium extracts and from ox-heart was inhibited more by mepacrine than by quinine. More recent work by Bovarnick, Lindsay, and Hellerman (1946b) showed that separated plasmodia, thoroughly exhausted of substrates by washing and preliminary incubation, were more sensitive to antimalarial drugs. The recovery of respiration after addition of glucose to the exhausted parasites was strongly inhibited by mepacrine, but no inhibition was observed with other substrates. Inhibition of glycolysis by mepacrine was antagonized by adenylic acid or adenosine triphosphate.

The present paper, reporting preliminary work on the metabolism of plasmodia, confirms generally the above reports on the glucose metabolism of malaria parasites, and presents fresh evidence of glycolysis by phosphorylation and on the mode of action of quinine and mepacrine.

Materials and Methods

Blood from chicks 2–4 weeks old, heavily infected (70–95 per cent of cells parasitized) with *P. gallinaceum*, was used as the source of parasite material. Since, in initial experiments, whole blood suspensions showed no response to added glucose, washed, parasitized red cells were used in most of the experiments described. The chicks were killed by placing in an atmosphere of CO_2, and blood drawn from the exposed heart was suspended in isotonic buffer (0.85 per cent NaCl, 100 ml. ; $M/15$ phosphate buffer, *p*H 7.3, 30 ml.) containing a little citrate to prevent coagulation. The suspension was centrifuged and the clear supernatant discarded ; the red cell layer was shaken with more isotonic buffer, re-centrifuged, and the red cells finally suspended in sufficient isotonic buffer to produce the required volume of suspension. This treatment removed all but a trace of indigenous glucose from the red cells.

Respiration experiments.—Oxygen consumption was measured in conventional Warburg respirometers, using 15 ml. flasks, maintained at 38° C. Determinations were carried out with a set of twelve respirometers, in duplicate, triplicate, or quadruplicate groups, according to the number of variants required. The amount of oxygen consumed was expressed in microlitres (μl.) per 1,000 million red blood cells, the percentage of cells parasitized being 70–95.

Glucose was determined in the incubates from the respirometer flasks by the method of Folin and Wu (1920). Pyruvic acid was determined by the modified method of Lu (1939) described by Umbreit, Burris, and Stauffer (1945), and lactic acid by the method of Barker and Summerson (1941).

Experiments on glucose phosphorylation.—Aliquots of a suspension of washed, parasitized red cells were incubated at 41° C. with a known amount of glucose, in contact with various inhibitors and antimalarial agents. A control aliquot was deproteinized immediately by adding an equal volume of ice-cold 12 per cent (w/v) trichloroacetic acid. The test aliquots were incubated for 4–5 hours, during which they were oxygenated, either by continuous bubbling, or at frequent intervals. At the end of the incubation period, the test aliquots were deproteinized, chilled in the cold-room, and the precipitated protein removed by centrifuging.

The clear supernatants were subjected to a complete analysis for glucose, phosphorylated intermediates, and pyruvic and lactic acids. Glucose was determined in dilutions of the supernatants by the method of Folin and Malmros (1929), and pyruvic and lactic acids by the methods already referred to. The remaining supernatant was adjusted to *p*H 8.2 and analysed for hexose phosphates, triose phosphates, and phosphoglyceric acid, using the experimental technique described by Umbreit, Burris, and Stauffer (1945). The amounts of the various intermediates were expressed as gram-molecules.

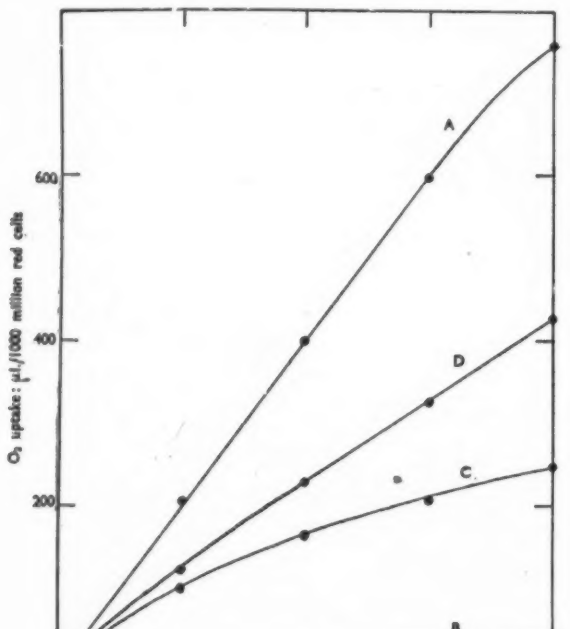

FIG. 1.—Oxygen uptake of normal and parasitized chick blood (single experiment). A.—Whole blood suspension (parasitized). B.—Whole blood suspension (normal). C.—Washed, parasitized red cells. D.—Washed, parasitized red cells + glucose (0.5 mg./ml.).

RESULTS

Respiration experiments

Though normal chick red cells showed a substantial oxygen uptake (compared with non-nucleated mammalian red cells), the respiration rate of heavily parasitized (96 per cent) chick red cells was about 15 times as rapid (Fig. 1). The respiratory rate of infected red cells (whole blood suspensions) increased proportionately with the number of cells parasitized (Table I). The oxygen uptake of infected whole blood suspensions was not increased, but actually depressed by adding glucose. If, however, the parasitized red cells were washed free from indigenous glucose, the oxygen uptake was reduced to 37 per cent (mean of 9 determinations; range 27.8–50.8 per cent) of that of the whole blood suspension (Fig. 1). The respiration of glucose-free infected cells was stimulated by added glucose, the recovery being graded

TABLE I

THE RELATION BETWEEN OXYGEN UPTAKE AND THE NUMBER OF RED CELLS PARASITIZED WITH *P. gallinaceum*

Whole blood suspensions; no added substrate

% cells parasitized	No. of respirometers	O_2 uptake (μl./hr./1000 million red cells)
normal	6	65.4
,,	6	48.4
,,	6	27.5
,,	5	36.2
,,	5	25.5
,,	2	19.5
,,	2	17.1
		Mean 34.2
30	5	77.8
30	5	89.0
44	3	146.0
80	5	250.0
85	5	296.0
88	2	235.0
96	6	314.0

TABLE II

EFFECT OF GRADED CONCENTRATIONS OF GLUCOSE ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS

Glucose added (mg./3 ml.)	% of O_2 uptake of whole blood suspension	% recovery of lost respiration
0.05	43.7	6.2
0.1	38.0	nil
0.5	57.2	28.7
0.5	56.5	35.5
0.5	52.1	26.3
		Mean 30.2
1.0	60.7	42.0
1.0	65.4	36.5
1.0	65.4	42.4
1.0	66.3	51.5
		Mean 43.1
2.0	81.7	71.8
2.0	73.0	45.1
		Mean 58.5
5.0	84.0	75.4
10.0	80.4	70.0

up to 5 mg./3 ml., when a maximum recovery of 75 per cent of the lost respiration rate was attained (Table II).

The quantity of oxygen consumed by plasmodia was not accounted for by the amount of glucose used; for example, when infected red cells were washed, the oxygen consumption was still about 40 per cent of that of the whole blood suspension, though the amount of available glucose was very small. Thus the oxygen consumption per molecule of glucose used was apparently increased (Table III). Again, since additional glucose did not increase the oxygen uptake of infected whole blood suspensions, the oxygen consumption and the amount of lactic acid formed per molecule of glucose appeared to decrease with increasing concentration of glucose (Table III). No pyruvic acid was formed. Table IV shows the course of quantitative utilization of glucose added to washed, parasitized red cells, and the amount of *extra* oxygen consumed. Neither lactic nor pyruvic acids accumulated in this instance.

TABLE III

QUANTITATIVE UTILIZATION OF GLUCOSE BY PLASMODIA

(a) Effect of graded concentrations on whole blood suspension.
(b) Effect of washing the infected red cells.

Preparation	Conc. of glucose g./100 ml.	Mols O_2 used per mol glucose consumed	mols lactic acid formed per mol glucose consumed
(a)	*added*		
Whole blood suspension	nil*	5.3	4.0
	0.01*	3.1	2.5
	0.05*	2.1	1.56
	0.10*	1.9	1.95
(b)	*present*		
Whole blood suspension	0.002	4.04	—
Washed cell suspension	0.00005	26.0	—

* + 0.02 g./100 ml. indigenous glucose.

TABLE IV

QUANTITATIVE UTILIZATION OF GLUCOSE BY WASHED, PARASITIZED RED CELLS

Time after adding glucose (min.)	μ-mols glucose used	μ-mols extra O_2 used	Mols O_2 used per mol glucose consumed
30	1.15	0.69	0.60
60	1.70	1.32	0.78
90	2.73	2.68	0.98
120	2.76	3.08	1.12

Substrates metabolized by plasmodium.—The oxygen uptake of washed, parasitized red cells was stimulated by glycerol, pyruvate, and lactate to the same extent as by glucose, but was only slightly stimulated by succinate and fumarate. Glutamate, aspartate, and tyrosine were inactive (Table V).

TABLE V

EFFECT OF ADDED SUBSTRATES ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS

Substrate added	Concentration (mg./3 ml.)	% stimulation of oxygen uptake
Glucose	1.0	75.6
Tyrosine	1.0	nil
Glucose	2.0	155
Pyruvate	2.0	161
Lactate	2.0	179
Succinate	2.0	13.5
Fumarate	2.0	12.9
Glucose	2.0	174
Glycerol	2.0	207
Glutamate ..	2.0	nil
Aspartate	2.0	nil

Effect of specific inhibitors.—Cyanide strongly inhibited the oxidation of glucose, lactate, and pyruvate by washed, parasitized red cells. Iodoacetic acid, in low concentration (1 in 30,000),

strongly inhibited glucose, but not pyruvate, oxidation; at higher concentration (1 in 6,000), the specificity of inhibition disappeared, and both substrates were equally inhibited (Table VI).

TABLE VI

EFFECT OF SPECIFIC INHIBITORS ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS ALONE, AND WITH ADDED SUBSTRATES

Inhibitor	Conc. (mg./3 ml.)	Percentage inhibition of O_2 uptake			
		No added substrate	Glucose	Pyruvic acid	Lactic acid
NaCN ..	0.5	80.4	87.5	93.7	87.7
Iodoacetic acid	0.5	36.0	42.0	86.0	74.6
	0.5		76.0	83.6	
	0.1		63.5	10.1	

Effect of antimalarial agents.—Quinine and mepacrine did not markedly inhibit the oxygen uptake of plasmodia either when respiring alone (washed, parasitized red cells), or in the presence of active substrates (Table VII). At fairly high concentration (1 in 6,000), there was little differentiation between the substrates, but at lower concentration (1 in 60,000), quinine showed a slightly greater inhibition of glucose and lactate oxidation, and mepacrine of lactate oxidation (Table VII).

TABLE VII

EFFECT OF QUININE AND MEPACRINE ON THE OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS ALONE, AND WITH ADDED SUBSTRATES

Anti-malarial agent	Conc. (mg./3 ml.)	Percentage inhibition of O_2 uptake after 2 hours			
		No added substrate	Glucose	Pyruvic acid	Lactic acid
Quinine	0.5	22.4	25.7	29.1	35.7
	0.05	6.0	17.3	2.3	18.9
Mepacrine	0.5	11.9	34.4	25.6	34.5
	0.05	9.0	2.0	5.8	14.6

At still lower concentration (1 in 300,000), the inhibitory effects, particularly of mepacrine, did not appear until the drugs had been incubated with the parasites for 2–6 hours (Table VIII).

Glucose phosphorylation

Changes in glucose, phosphorylated intermediates, and lactic and pyruvic acids during incubation of infected red cell suspensions were expressed as molecules per 100 molecules of glucose used by uninhibited, parasitized red cells. Table IX shows the comparison between the glucose metabolism of uninfected chick red cells and parasitized red cells. Parasitized cells used about four times as much glucose as normal red cells, and, with parasitized cells, a large amount of lactic acid accumulated (0.5 to 1.5 molecule per molecule of glucose used). Glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate were metabolized more rapidly in parasitized cells than in normal cells, but triose phosphate and pyruvic acid accumulated to a greater extent than in uninfected red cells. These observations showed that active glucose phosphorylation was proceeding more rapidly in infected red cell incubates than in normal cell incubates.

TABLE VIII

INHIBITION OF OXYGEN UPTAKE OF WASHED, PARASITIZED RED CELLS BY LOW CONCENTRATIONS OF QUININE AND MEPACRINE

Anti-malarial agent	Substrate	Percentage inhibition after incubation for		
		2 hr.	4 hr.	6 hr.
Quinine (0.01 mg./3 ml.)	none	nil	5.4	16.8
	glucose	9.4	15.7	20.0
Mepacrine (0.01 mg./3 ml.)	none	nil	nil	3.1
	glucose	nil	6.8	9.8

Effect of specific inhibitors.—Iodoacetic acid completely inhibited the utilization of glucose and accumulation of lactic acid, and, in the accumulation of hexose and triose phosphates, showed the typical effects of phosphoglyceraldehyde dehydrogenase blockage. Cyanide inhibited the utilization of glucose by 40 per cent, but the amount of lactic acid formed was greater than in the controls. Cyanide stimulated the utilization of phosphorylated intermediates and of pyruvic acid, as shown by a decrease during the incubation period.

Effect of antimalarial agents.—Quinine inhibited glucose utilization more powerfully than mepacrine did. The most marked effect of mepacrine was the large accumulation of adenosine triphosphate (ATP), indicating inhibition of the enzyme hexokinase by which glucose is initially phosphorylated. Quinine also inhibited hexokinase, but to a smaller extent than mepacrine. Both drugs caused accumulation of glucose-6-phosphate, but not to the same extent as iodoacetic acid. In the presence of quinine, pyruvic acid accumulated; mepacrine reduced the accumulation of lactic acid more than quinine did.

TABLE IX

EFFECT OF SPECIFIC INHIBITORS AND ANTIMALARIALS ON GLUCOSE PHOSPHORYLATION BY WASHED CHICK RED CELLS INFECTED WITH *P. gallinaceum*

Figures denote the mean numbers of μ mols of the substrates formed (+) or used (−) per hour per 100 μ mols of glucose consumed by the control suspension

No. of expts.	Inhibitor	Molar conc. of inhibitor	Glucose	ATP	Glucose-1-phosphate	Glucose-6-phosphate	Fructose-6-phosphate	Fructose-1, 6-diphosphate	Triose phosphates	Phosphoglyceric acid	Phosphopyruvic acid	Pyruvic acid	Lactic acid
1	(Normal blood)	—	−23	+1.66	+1.16	+1.19	+0.24	+0.65	+0.66	nil	nil	+1.51	nil
4	(No inhibitor)	—	−100	+0.79	+6.99	−3.81	−0.05	−0.01	+4.47	−1.29	nil	+5.19	+115
3	Iodoacetic acid	1.08×10^{-3}	+4	−0.42	+7.90	+10.22	+0.70	+0.23	+4.67	+1.00	+1.07	+2.01	+6
	(Controls)	—	−100	+1.05	+9.32	−0.17	+0.19	+0.15	+5.96	−1.10	nil	+6.04	+143
2	NaCN	4.08×10^{-3}	−60	−0.93	+4.26	−4.13	−0.07	+0.61	+0.53	−1.56	+5.84	−0.42	+90
	(Controls)	—	−100	+0.66	−0.92	−9.80	−0.30	−0.25	+0.40	−1.10	nil	+5.02	+69
3	Quinine	5.05×10^{-4}	−42	+2.39	+5.97	+1.14	−0.18	+0.41	+1.87	−0.28		+5.37	+94
	(Controls)	—	−100	+0.61	+9.93	−3.47	−0.11	−0.02	+5.69	−1.60		+4.44	+118
3	Mepacrine	3.94×10^{-4}	−79	+6.46	−0.91	+2.26	−0.17	+0.47	+3.48	−0.30	nil	+1.02	+57
	(Controls)	—	−100	+0.61	+9.93	−3.47	−0.11	−0.02	+5.69	−1.60	nil	+4.44	+118

Discussion

Although the malaria parasite utilizes glucose, its metabolism of this substrate is comparatively slow; thus, in whole blood suspensions, sufficient indigenous glucose is present to last the parasites for several hours, and added glucose produces no stimulation of respiratory activity. That glucose is a necessary substrate for the full activity of plasmodia is, however, shown by the fact that infected red cells lose 60 per cent of their respiratory activity when washed free from glucose, and that most of this lost activity can be restored by adding glucose to the washed cell suspension.

The respiration rate of washed, parasitized red cells can be stimulated by substrates other than glucose. As observed by previous workers (Speck, Moulder and Evans, 1946), lactic and pyruvic acids are as active as, or slightly more active than, glucose in this respect (see, however, the next paragraph). Succinic and fumaric acids show a small but definite stimulatory effect. From these observations it is apparent that the carbohydrate metabolism of plasmodia proceeds to some end-point beyond lactic and pyruvic acids. The stimulation by succinate and fumarate suggests that pyruvic acid is metabolized *via* the Krebs citric acid cycle. Using parasites freed from red cells, Speck, Moulder, and Evans (1946) obtained much greater stimulation with succinate.

Analysis of parasitized red cell incubates for phosphorylated intermediates confirmed the view of previous workers that the initial stages of glucose metabolism by plasmodia follow the so-called Embden-Meyerhof-Parnas system characteristic of yeast and muscle metabolism. Since considerable quantities of lactic acid accumulated in these incubates, it seems that the conversion of glucose to pyruvic acid is more rapid than the subsequent utilization of pyruvate, the excess pyruvate being reduced to lactate. McKee *et al.* (1946) reported that lactate was produced six times as rapidly as it was utilized.

The amount of oxygen utilized by plasmodia bears no constant relation to the amount of glucose used. In parasitized red cell incubates respiring in the presence of glucose, the amount of oxygen consumed is at first less than one molecule per molecule of glucose used, but gradually increases to more than one molecule during the incubation period. This gradual increase in oxygen consumption probably indicates the "starting up" of additional oxidation systems as the metabolism of the initial quantity of glucose reaches the various stages. The fact that it takes several hours to build

up to the full rate of oxygen consumption emphasizes the sluggishness of carbohydrate metabolism in plasmodia.

Oxygen enters into the metabolic system of plasmodia at two points at least; firstly in the oxidation of glucose to pyruvic acid, and secondly in the subsequent oxidation of the pyruvate. Since the first oxidation (which takes place at the conversion of phosphoglyceraldehyde to phosphoglyceric acid) is inhibited by iodoacetic acid, it is concluded that it takes place through a chain of reducing reactions catalysed by dehydrogenase systems. However, the final connection with atmospheric oxygen is probably made through the cytochrome oxidase system, since glucose oxidation is as powerfully inhibited by cyanide as pyruvate oxidation. Furthermore, in the presence of cyanide, glucose and the phosphorylated intermediates are rapidly converted to lactic acid, which is an anaerobic reaction requiring no oxygen. Pyruvate oxidation is not appreciably inhibited by iodoacetic acid, but is strongly inhibited by cyanide, and is therefore entirely dependent on the cytochrome oxidase system.

Considerable quantities of glucose-1-phosphate (an intermediate of glycogen, but not of glucose, metabolism) accumulated in parasitized red cell incubates. This accumulation probably resulted from a "feed-back" from glucose-6-phosphate through the reversible phosphoglucomutase reaction. This view is strengthened by the observation that accumulation of glucose-1-phosphate was increased in the presence of iodoacetic acid, where the later stages of normal glucose phosphorylation were blocked.

Parasitized red cells from which all traces of indigenous glucose have been removed by repeated washing still show an appreciable oxygen uptake, which is still proceeding after 6 hours' incubation. It is generally believed that plasmodia can metabolize substrates other than carbohydrate, but, in view of the slowness of glucose metabolism, the possibility cannot be ignored that sufficient quantities of the glucose intermediate compounds might remain in the parasites or red cells after washing to account for the "residual" respiration observed. The oxygen uptake of washed, parasitized red cells was not stimulated by amino-acids (glutamate, aspartate or tyrosine) nor could any change in non-protein or ammonia nitrogen be detected in the incubates. This is not, however, in agreement with the results of previous workers (Moulder and Evans, 1946), who found both that amino-acids were utilized, and that non-protein and ammonia nitrogen were produced by plasmodia.

Having obtained some indication of the course of carbohydrate metabolism in plasmodium, we may consider at what points in this system quinine and mepacrine exert inhibitory actions. In agreement with the observations of previous workers (Silverman *et al.*, 1944) it was noted that neither drug, in low concentration, inhibited the oxygen uptake of parasitized red cells until it had been in contact with the cells for 2–6 hours; even then, the inhibition was not very pronounced.

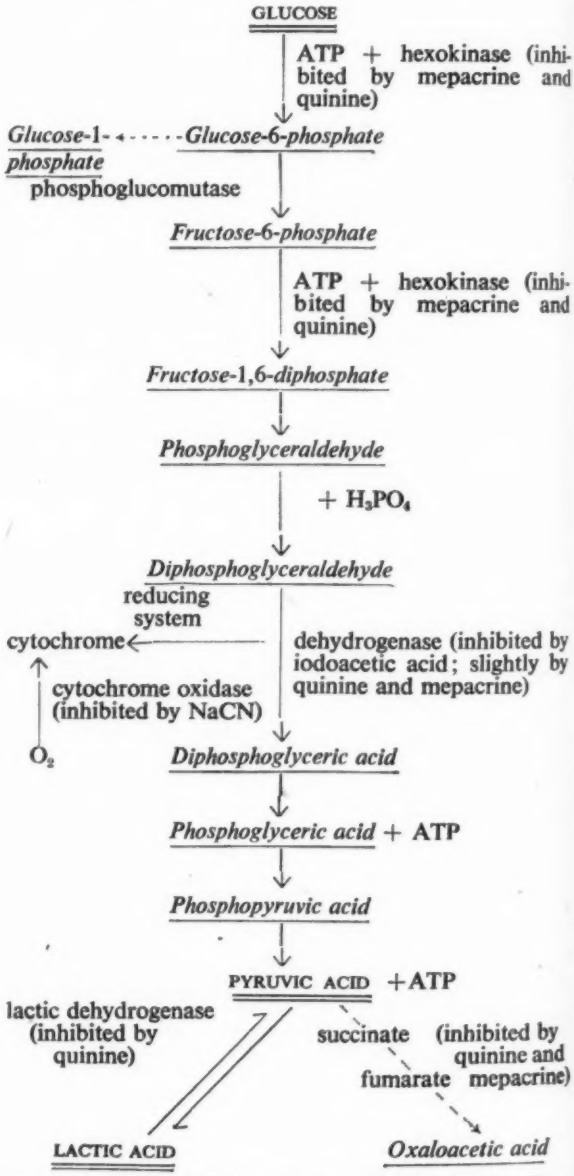

FIG. 2.—Scheme of glucose metabolism of *P. gallinaceum*, with indications of the points of action of quinine and mepacrine.

Bovarnick *et al.* (1946b) reported that mepacrine inhibition of plasmodia could be reversed by ATP, and Speck and Evans (1945b) showed that both quinine and mepacrine inhibited yeast hexokinase. In the present investigation, the analyses showed that, while quinine inhibited glucose utilization to a greater degree than mepacrine, the latter compound showed a more pronounced inhibition of hexokinase. In the presence of mepacrine, the accumulation of glucose-1-phosphate was inhibited, presumably because of the formation of glucose-6-phosphate was reduced. Both drugs showed some iodoacetate-like inhibitory effects, but these were not well marked. Quinine caused lactate and pyruvate to accumulate, and therefore inhibited lactate as well as pyruvate oxidation. These possible points of action are indicated in Fig. 2.

From the above observations, it is clear that quinine and mepacrine exert inhibitory activity at several points in the metabolic scheme of plasmodia. With an organism possessing a comparatively complicated metabolism, it is necessary that a therapeutic agent should have this capacity for multiple attack in order to be effective against the organism. For instance, trivalent arsenical compounds, which inhibit only one type of enzyme, the so-called -SH enzymes, are active against certain trypanosomes which possess a simpler type of metabolism, but are inactive against plasmodia, in which alternative metabolic paths probably exist, or a greater variety of substrates can be utilized. The present investigation has given indications of the points of action of antimalarial drugs in one metabolic system — carbohydrate metabolism. Further studies are necessary to find what proportion of the total inhibitory activity takes place at the different points, and, indeed, whether the greater part of the inhibition does take place against the carbohydrate metabolism, or against other metabolic functions.

Summary

1. Investigations on the oxygen uptake of *Plasmodium gallinaceum* show that washed, parasitized chick red cells can oxidize glucose, glycerol, lactate, and pyruvate, but not glutamate, aspartate, or tyrosine. Stimulation of oxygen uptake by succinate and fumarate indicates that pyruvate metabolism proceeds *via* the Krebs citric acid cycle.

2. Observation of the changes in phosphorylated intermediates in washed, parasitized red cell incubates confirms the view that glucose metabolism in plasmodia proceeds *via* the Embden-Meyerhof-Parnas system characteristic of yeast and muscle metabolism.

3. The possibility of metabolic systems in plasmodia other than carbohydrate is discussed.

4. Quinine and mepacrine exert inhibitory activity at several points in the glucose metabolism of plasmodia. Quinine inhibits hexokinase and phosphoglyceraldehyde dehydrogenase moderately, and possibly lactic dehydrogenase and pyruvate oxidation. Mepacrine inhibits hexokinase strongly, phosphoglyceraldehyde dehydrogenase moderately, and probably pyruvate oxidation.

The author is indebted to Mr. L. G. Goodwin (Pharmacology Dept.) for supplying chicks infected with *P. gallinaceum*, and to Messrs. P. A. Hankin and R. W. Neville for valuable technical assistance.

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THE GLUCOSE METABOLISM OF *TRYPANOSOMA EVANSI* AND THE ACTION OF TRYPANOCIDES

BY

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It has long been recognized that trypanosomes are dependent on an adequate supply of glucose for the maintenance of their metabolic processes (Yorke, Adams, and Murgatroyd, 1929; Geiger, Kligler, and Comaroff, 1930; von Brand, 1933). Later work has given some indication of the mechanism of the glucose metabolism of various species of the parasite, and the nature of the end-points formed. Reiner and Smythe (1934) showed that the end-point of glucose metabolism in *T. equiperdum* was pyruvic acid, and the same group of workers (Reiner, Smythe, and Pedlow, 1936) showed that *T. lewisi* metabolized glucose more completely to yield formic, acetic and succinic acids, ethyl alcohol and carbon dioxide. Fulton and Stevens (1945) reported that the products of metabolism of *T. rhodesiense* were succinic, pyruvic, lactic, acetic and formic acids, glycerol, ethyl alcohol, and carbon dioxide.

Regarding the respiratory activity of trypanosomes, Christophers and Fulton (1938) showed that oxygen consumption by *T. rhodesiense* was entirely dependent on the presence of glucose, and that 1 molecule of glucose required 1 molecule of oxygen. They also demonstrated the presence of dehydrogenase systems in trypanosome metabolism, and the absence of inhibition by cyanide. Reiner and Smythe (1934) reported that *T. equiperdum* produced very little carbon dioxide in the absence of bicarbonate, and that 1.80 molecules of acid (mostly pyruvic) were produced from the metabolism of 1 molecule of glucose. These workers also showed that trypanosomes could metabolize glycerol as well as glucose, but only under aerobic conditions. More recent work by Searle and Reiner (1940, 1941) has demonstrated the importance of carbon dioxide as an activator of anaerobic glycolysis in trypanosomes.

These investigations show clearly the nature of the end-products of glucose metabolism in various species of trypanosomes, but give little indication of the intermediate processes by which these products are formed. Indirect evidence, such as the ability of glycerol to replace glucose, suggests that the intermediate metabolism of trypanosomes follows the typical phosphorylation course associated with yeast and muscle metabolism. However, only in one instance, a recently published paper by Chen and Geiling (1946), has any direct evidence of glucose phosphorylation been demonstrated. These workers have shown that lysed trypanosomes will transform glucose to fructose-1,6-diphosphate and triose phosphates, and will oxidize phosphoglyceraldehyde to phosphoglyceric acid.

The investigations described in this communication corroborate the findings of other workers regarding the respiratory activity of trypanosomes, and provide further evidence that glycolysis proceeds *via* the typical chain of phosphorylation reactions. Some preliminary indications are given of the points of attack by trypanocidal agents, on which there are no previous reports.

Materials and Methods

The organism was a strain of *Trypanosoma evansi* isolated from a camel in the Sudan in 1938, and maintained in mice, in which it produces heavy, acute blood infections. Infected blood was obtained from the exposed hearts of mice killed by placing them in an atmosphere of carbon dioxide.

Respiration experiments.—Oxygen consumption was measured in conventional Warburg constant volume respirometers, using 15-ml. flasks maintained at 38° C. A set of twelve respirometers was run in duplicate, triplicate, or quadruplicate groups, according to the number of variants required. Since normal mouse blood showed only a negligible oxygen uptake, whole blood containing the trypanosomes was usually used, diluted with "isotonic buffer" (0.85 per cent NaCl, 100 ml.; *M*/15 phosphate buffer, *p*H 7.3, 30 ml.) containing a little citrate to prevent clotting. The concentration of trypanosomes was 10 to 100 million per flask. In a few experiments where blood-free trypanosomes were required, the parasites were separated by adding an equal volume of distilled

water to the blood-buffer suspension, which contained 0.05 per cent glucose. The resultant lowering of tonicity lysed the red cells of the blood, but did not disrupt the trypanosomes, which were centrifuged down, washed free from haemoglobin with isotonic buffer-glucose, and finally resuspended in glucose-free isotonic buffer.

Glucose was determined in the incubates from the respirometer flasks by the method of Folin and Wu (1920). Pyruvic acid was determined by the modified method of Lu (1939) described by Umbreit, Burris, and Stauffer (1945), and lactic acid by the method of Barker and Summerson (1941).

Experiments on glucose phosphorylation.—Mouse blood heavily infected with trypanosomes was diluted with isotonic buffer containing a known concentration of glucose. An aliquot of the suspension was deproteinized immediately by adding an equal volume of ice-cold 12 per cent (w/v) trichloroacetic acid. Similar aliquots were incubated at 38° C. for 40–100 min., either alone, or in contact with inhibitors or trypanocides. These were then treated with trichloroacetic acid, chilled in the cold-room, and the precipitated protein removed by centrifuging. The concentration of trypanosomes was 10 to 100 million per aliquot.

The clear supernatants were subjected to a complete analysis for glucose, phosphorylated intermediates, pyruvic and lactic acids. Glucose was determined in dilutions of the supernatants by the method of Folin and Malmros (1929); pyruvic and lactic acids by the methods already referred to. The remaining supernatant was adjusted to *p*H 8.2 and analysed for hexose phosphates, triose phosphates, phosphoglyceric acid and phosphopyruvic acid using the experimental technique described by Umbreit, Burris, and Stauffer (1945). The amounts of the various intermediates were expressed in molecular proportions (μ moles).

The trypanocides investigated were: phenylarsine oxide—trivalent arsenical; tryparsamide—pentavalent arsenical; undecane diamidine—straight chain diamidine; stilbamidine—aromatic diamidine.

Samples of phenylarsine oxide and tryparsamide were kindly supplied by Dr. T. Dewing of the Wellcome Chemical Laboratories, Dartford.

Results

Respiration experiments

Fig. 1 shows the oxygen uptake of an infected blood suspension containing increasing concentrations of glucose. The initial respiratory rate was the same for all concentrations, but the oxygen uptake ceased abruptly as soon as all the glucose had been used up. This experiment showed clearly the dependence of trypanosomes on an adequate supply of glucose. The more gradual falling off of respiratory rate in the presence of excess glucose was at first presumed to be the result of toxic action by accumulated end-products. However, subsequent experiments showed that the *p*H of the medium was not appreciably lowered during the experiment, nor was added pyruvate (the chief end-product) toxic to the trypanosomes. The slowing down of respiratory rate may therefore be caused by exhaustion of available nutritional factors in the parasites or host blood.

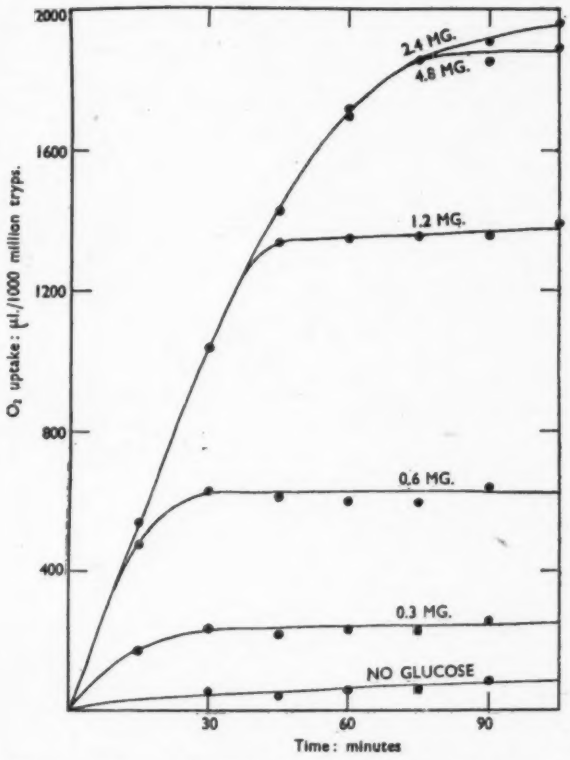

FIG. 1.—Effect of increasing amounts of glucose on the duration of respiration of mouse blood suspension containing *T. evansi*.

The chief end-product of glycolysis by *T. evansi* was pyruvic acid, which accumulated finally in the proportion of 1.75 molecules per molecule of glucose used, one molecule of oxygen being used up in the reaction (Fig. 2).

The rate of oxygen uptake of suspensions of trypanosomes separated from the blood of the host was reduced to 50 per cent of that of the original whole blood suspension. Nearly 75 per cent of the lost respiratory activity was recovered by adding 5 per cent of mouse plasma to the separated parasites (Table I), but addition of lysed mouse red cells had no stimulatory effect. It may be, therefore, that the trypanosome relies on the plasma of the host for many of the nutritional factors required for its metabolism. Individual factors, including pantothenic, nicotinic, adenylic acids, thiamin, riboflavin, pyridoxin, and inositol were

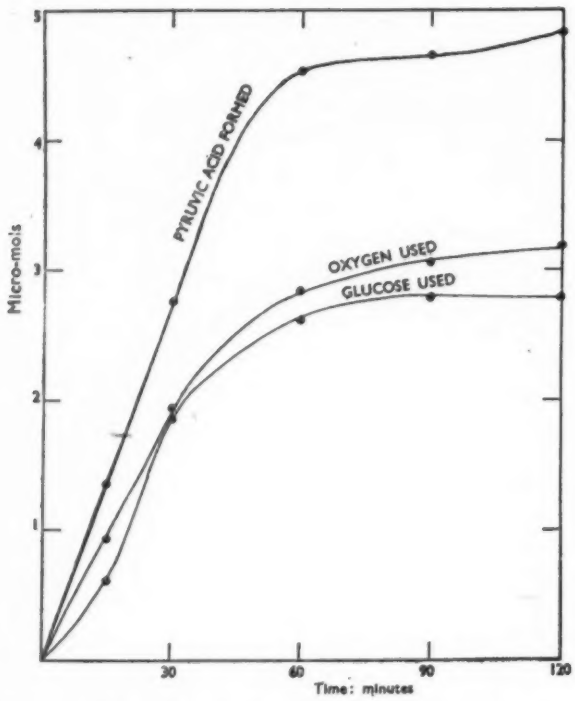

FIG. 2.—The utilization of glucose and oxygen and the formation of pyruvic acid by *T. evansi* (whole blood suspension).

TABLE I

STIMULATORY EFFECT OF MOUSE PLASMA ON THE OXYGEN UPTAKE OF SEPARATED, WASHED TRYPANOSOMES

Washed parasites suspended in isotonic buffer + 0.05 per cent glucose. O_2 uptake compared with that of the original whole blood suspension

Expt.	% plasma added	% of "normal" respiration rate	% recovery
156	none	52.5	—
	5.0	87.8	74.2
157	none	45.2	—
	3.33	79.0	61.5
	1.66	68.5	42.7
	0.66	55.5	18.7
	0.16	55.2	18.4

investigated, but none increased significantly the respiratory rate of separated trypanosomes.

Trypanosome suspensions respired in the presence of glycerol as readily as with glucose. One molecule of oxygen was used up per molecule of glycerol, and one molecule of pyruvic acid was formed. Lactate, fumarate, succinate, glutamate, and aspartate were not metabolized. Pyruvate produced a small additional oxygen uptake, particularly in the presence of glucose. With glycogen, a slow, prolonged oxygen uptake was observed. It was found, however, that glycogen was slowly hydrolysed to glucose by normal mouse blood. The respiration of the trypanosomes in the presence of glycogen therefore resulted from the utilization of this glucose as it was produced, and not from a direct metabolism of the polysaccharide.

TABLE II

EFFECT OF SPECIFIC INHIBITORS ON THE O_2 UPTAKE OF TRYPANOSOMES

Whole blood suspensions containing added glucose

Inhibitor	Concentration		% inhibition
	μg./3 ml.	molar	
Iodoacetic acid ..	50.0	8.98×10^{-4}	92.0
	10.0	1.79×10^{-4}	89.3
	2.0	3.58×10^{-5}	53.3
	0.4	7.71×10^{-7}	4.6
	0.08	1.43×10^{-7}	nil
Sodium fluoride ..	50.0	3.97×10^{-4}	41.5
	10.0	7.94×10^{-5}	13.0
	2.0	1.59×10^{-5}	2.7
	0.4	3.18×10^{-6}	nil
Sodium cyanide ..	50.0	3.4×10^{-4}	10.3
	500.0	3.4×10^{-3}	8.1

Effect of specific inhibitors.—The inhibitory effects of iodoacetic acid, NaCN, and NaF on the oxygen uptake of infected blood suspensions are shown in Table II. Iodoacetic acid and NaF were powerful inhibitors of trypanosome respiration. Cyanide had little effect on the oxygen uptake, but the amount of pyruvic acid formed was reduced to one molecule per molecule of glucose (Fig. 3).

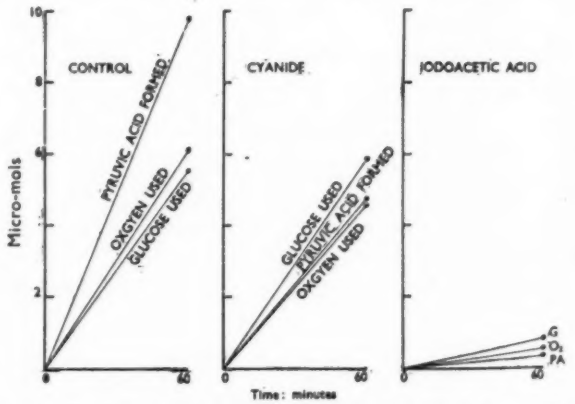

FIG. 3.—Effect of NaCN and iodoacetic acid on the utilization of glucose and oxygen and the formation of pyruvic acid by *T. evansi* (whole blood suspension).

Effect of trypanocides.—The oxygen uptake of trypanosome suspensions was almost completely inhibited by concentrations of 1 in 300,000 of phenylarsine oxide, but was only slightly inhibited by 1 in 30,000 of tryparsamide. Stilbamidine had little inhibitory effect even in concentrations as high as 1 in 6,000. Undecane diamidine, however, produced 50 per cent inhibition of oxygen uptake at 1 in 30,000 concentration, but no further inhibition was obtained on increasing the concentration above this level (Table III).

TABLE III

EFFECT OF TRYPANOCIDES ON THE O_2 UPTAKE OF TRYPANOSOMES

Whole blood suspensions containing added glucose

Trypanocide	Concentration		% inhibition
	μg./3 ml.	molar	
Phenylarsine oxide (Trivalent As)	10.0	1.98×10^{-5}	81.3
	5.0	9.93×10^{-6}	49.0
	2.0	3.97×10^{-6}	34.0
	0.5	9.93×10^{-7}	14.6
	0.1	1.98×10^{-7}	5.7
Tryparsamide .. (Pentavalent As)	100.0	1.09×10^{-4}	1.70
	10.0	1.09×10^{-5}	4.78
Stilbamidine ..	500.0	6.32×10^{-4}	3.65
	100.0	1.26×10^{-4}	nil
	100.0	1.26×10^{-4}	2.98
	10.0	1.26×10^{-5}	2.08
Undecane diamidine di-HCl	500.0	5.35×10^{-4}	48.5
	100.0	1.07×10^{-4}	51.5
	100.0	1.07×10^{-4}	48.0
	10.0	1.07×10^{-5}	32.4

Phosphorylated intermediate analyses

In these experiments, the standard of activity was taken as the amount of glucose utilized by uninhibited trypanosome suspensions, and the changes in concentration of the intermediate and end-products were expressed as the mean number of molecules used (−) or produced (+) per 100 molecules of glucose consumed by uninhibited, infected blood suspensions.

Glucose phosphorylation by normal and infected blood.—Table IV shows that trypanosome-infected blood used more than 10 times as much glucose as did normal blood ; that glucose-6-phosphate was

TABLE IV

EFFECT OF SPECIFIC INHIBITORS AND TRYPANOCIDES ON GLUCOSE PHOSPHORYLATION BY *T. evansi*

Figures denote the mean numbers of μ moles of the substrates formed (+) or utilized (−) per hour per 100 μ moles of glucose consumed by the control suspension (uninhibited trypanosome-blood suspension)

No. of expts.	Inhibitor	Molar conc. of inhibitor	Glucose	ATP	Glucose-1-phosphate	Glucose-6-phosphate	Fructose-6-phosphate	Fruct.-1, 6-diphosphate	Triose phosphates	Phospho-glyceric acid	Phospho-pyruvic acid	Pyruvic acid	Lactic acid
1	(Normal blood)	—	−7	+2.9	+1.2	−0.8	+0.49	+0.34	+0.3	−1.9	+0.18	+4	+13.7
4	(No. inhib.)	—	−100	+6.6	+1.4	−26.7	+0.79	−0.17	+10.7	−4.1	+3.65	+174	−0.21
4	Iodoacetic acid	5.38×10^{-4}	−38	−1.2	+2.2	−13.8	+0.42	+0.38	−2.0	−1.1	+2.55	+12	nil
	(Controls)	—	−100	+6.6	+1.4	−26.7	+0.79	−0.17	+10.7	−4.1	+3.65	+174	−0.21
2	NaCN	4.08×10^{-3}	−173	+9.1	−0.6	−16.8	+0.69	−0.02	+0.5	+0.2	+1.53	+203	+0.7
	(Controls)	—	−100	+5.8	−0.6	−20.3	+1.23	−0.01	−0.1	−2.9	+3.65	+159	−3.8
2	Phenylarsine oxide	5.95×10^{-4}	−24	+10.8	+4.1	−20.9	+0.07	−0.15	+2.8	−20.8		+7	+3.5
	(Controls)	—	−100	+7.4	+3.4	−33.1	+0.36	−0.35	+21.5	−5.4		+189	+3.3
1	Stilbamidine ..	3.79×10^{-4}	−78	−4.1	−10.7	+10.5	+0.96	−0.70	+6.4	−8.9		+202	+8.8
	(Controls)	—	−100	+11.5	+6.8	−1.2	+0.72	−0.70	+21.0	−5.7		+215	+12.3
2	Undecane diamidine	6.4×10^{-4}	−148	+7.2	−0.7	−9.4	+1.36	+0.38	+3.2	−0.7	+5.36	+190	−0.6
	(Controls)	—	−100	+5.8	−0.6	−20.3	+1.23	+0.01	−0.1	−2.9	+3.65	+159	−3.8

very rapidly utilized by infected blood; and that glucose-1-phosphate, triose phosphate, phosphopyruvic acid and particularly pyruvic acid accumulated in parasitized blood incubates. Adenosine triphosphate (ATP) accumulated more rapidly in infected than in normal blood. The mean amount of pyruvic acid formed was 1.74 molecules per molecule of glucose used, which agrees with the results of the respiration experiments. Normal blood produced practically no pyruvic acid, but some lactic acid accumulated.

Effect of specific inhibitors.—In the presence of iodoacetic acid, glucose utilization was strongly inhibited, ATP was consumed, and glucose-6-phosphate disappearance was retarded, while hexose diphosphate accumulated. These observations are in accordance with the accepted view that iodoacetic acid blocks the dehydrogenase reaction which promotes the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. Cyanide actually increased the amount of glucose utilized, but reduced the proportion of pyruvic acid formed. It had little effect on the intermediates of the system.

Effect of trypanocides.—Of the trypanocides investigated, phenylarsine oxide had the most striking effect. In the presence of this compound, very little glucose was utilized, and ATP accumulated in the incubate. This suggests an inhibition of hexokinase, since the ATP released by dephosphorylation was not being re-utilized to phosphorylate more glucose. The intermediates beyond the "block" were rapidly consumed.

Undecane diamidine behaved similarly to cyanide in that it increased the glucose utilization and reduced the proportion of pyruvic acid formed. Stilbamidine inhibited glucose utilization slightly, but increased the proportion of pyruvic acid produced to 2.6 molecules. Its effect on the intermediate metabolism resembled that of iodoacetic acid, though glucose utilization was not inhibited to the same extent.

Discussion

The preliminary observations on *T. evansi* showed that suspensions of the parasites metabolized glucose and glycerol exclusively, the oxygen uptake ceasing abruptly as soon as the added substrate was consumed. The end-point of the metabolism of either substrate was pyruvic acid, and quantitatively the gross reactions taking place conformed fairly closely to the equations

$$\underset{\text{glucose}}{C_6H_{12}O_6} + O_2 \longrightarrow 2\underset{\text{pyruvic acid}}{C_3H_4O_3} + 2H_2O$$

and $$\underset{\text{glycerol}}{C_3H_8O_3} + O_2 \longrightarrow C_3H_4O_3 + 2H_2O$$

These observations are similar to those reported for *T. equiperdum* (Reiner and Smythe, 1934). No other substrates were utilized by *T. evansi*. Polysaccharides (glycogen) were not broken down directly, and none of the intermediates associated with the subsequent utilization of pyruvic acid (succinate and fumarate), nor amino-acids, stimulated the respiratory activity of the trypanosomes. The metabolic activity of this trypanosome therefore appears to be limited to the conversion of hexoses to pyruvic acid, since glycerol can be regarded as an intermediate of this process.

The next step was to examine the intermediate stages of this reaction. In most living cells, glucose breakdown proceeds *via* the so-called Embden-Meyerhof-Parnas scheme of reactions (Fig. 4) which operates through a chain of phosphorylated hexose intermediates. Previous work on trypanosomes suggested that the metabolism of these organisms followed this scheme. In the present work, analysis of trypanosome suspensions before and after a period of incubation with glucose showed that active changes in the levels of phosphorylated intermediates had taken place. These changes were much greater in parasitized blood than in normal mouse blood. Addition of iodoacetic acid slowed down the rate of utilization of the hexose phosphates as well as the oxygen consumption of the parasites. From these observations it is concluded that the glucose metabolism of the trypanosomes followed the Embden-Meyerhof-Parnas scheme.

The only point at which atmospheric oxygen enters into the above scheme is in the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. Biological oxidations may be accomplished either by transferring oxygen through a chain of oxidation reactions to the compound to be oxidized, or by transferring hydrogen from the compound through a chain of reducing actions to a point where it can be oxidized to water by atmospheric oxygen. Oxidation systems (usually including the cytochrome system) are inhibited by cyanide; dehydrogenase systems by iodoacetic acid. Since the oxygen uptake of trypanosomes was inhibited by iodoacetic acid, but not by cyanide, it is concluded, in agreement with Christophers and Fulton (1938), that only dehydrogenase systems operate in their utilization of oxygen.

In any metabolic system, certain co-factors are necessary for the functioning of the enzyme reactions involved. The factors required by trypanosomes may be obtained from the plasma of the

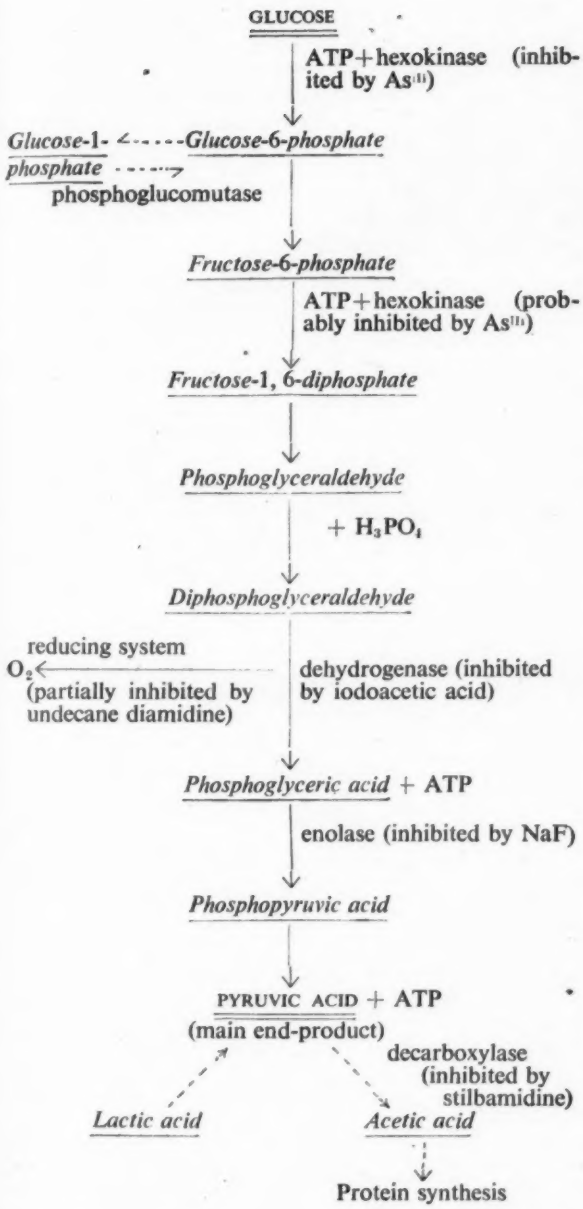

FIG. 4.—Scheme of glucose metabolism of *T. evansi*, with indications of the points of action of trypanocides.

host, since the respiration rate of trypanosomes separated from plasma was reduced by half, and the activity could be restored by adding normal mouse plasma. Other explanations, such as osmotic disturbances and surface phenomena, could account for this loss of metabolic activity, but the fact that only 5 per cent of plasma was required to restore most of the lost respiratory rate would favour the nutritional factors as the most likely explanation.

Although the above equation for the oxidation of glucose to pyruvic acid was fairly well satisfied, it was noticed repeatedly that only 1.75 molecules of pyruvic acid accumulated, instead of the theoretical 2 molecules, and that a little more than one molecule of oxygen was utilized. This suggests that a little of the pyruvate is oxidized by the trypanosomes, and might be the source of the protein which must be synthesized for the multiplication of the parasites. Some indication of the mode of metabolism of the quarter molecule of pyruvic acid was provided by the observation that cyanide decreased the amount of pyruvic acid which accumulated, relative to the amount of glucose used up. Tauber (1938) reported that cyanide was an activator of co-carboxylase, and it is probable, therefore, that the pyruvate undergoes preliminary decarboxylation. The fact that trypanosomes produce a small amount of respiratory CO_2 (Reiner and Smythe, 1934 ; Christophers and Fulton, 1938) supports this theory, since the reaction ending in pyruvic acid produces no CO_2.

It is now possible to consider at what points in this metabolic scheme the various types of trypanocides exert their action. It is clear from the results that trivalent arsenicals strongly inhibit the hexokinase reaction, and therefore block the utilization of glucose at the first stage, conversion to the 6-phosphate. This observation agrees with the report of Dixon and Needham (1946) on the action of arsenical vesicants on the glucose metabolism of skin. Pentavalent arsenicals showed no inhibition of trypanosome respiration, which is in accordance with the view that these compounds must first be reduced *in vivo* to the trivalent state before becoming active.

The point of action of the diamidines was less obvious than that of arsenic, but it appears that the straight chain compounds (undecane diamidine) differ in action from the aromatic compounds (stilbamidine). The former compound behaved similarly to cyanide in decreasing the amount of pyruvate formed, while stilbamidine had the reverse effect of increasing the pyruvate accumulation. Though stilbamidine caused striking changes in the levels of phosphorylated intermediates, it did not interfere to any extent with the utilization of glucose or oxygen during the 60 to 90 minute period of the manometric experiments. Its trypanocidal action may therefore result from the inhibition of pyruvate metabolism, causing ultimate suppression of growth. These conclusions are in agreement with the obser-

vations of Lourie and Yorke (1937) that this type of compound (synthalin) only showed trypanocidal activity *in vitro* after at least 24 hours' incubation. The point of action of undecane diamidine is not clear, since intermediate glucose metabolism is not greatly influenced by the compound. It did, however, cause a 50 per cent reduction in oxygen uptake, so that its point of action may lie somewhere in the dehydrogenase system (Fig. 4).

Two compounds which do not appear to fit directly into the metabolic scheme of trypanosomes require some consideration. The first one, glucose-1-phosphate, is not an intermediate of glucose but of glycogen phosphorylation. Although it was shown that trypanosomes do not metabolize glycogen directly, an increase in glucose-1-phosphate was observed in some cases in uninhibited incubates and in the presence of inhibitors blocking the normal metabolic chain. In iodoacetic acid inhibition, where the blockage occurs beyond the glucose-6-phosphate stage (Fig. 4), accumulation of glucose-1-phosphate might result from a " feed-back " from the 6-phosphate *via* the reversible phosphoglucomutase reaction. With phenylarsine oxide, however, where the greatest accumulation of glucose-1-phosphate occurred, this " feed-back " could not operate, since the formation of the 6-phosphate itself from glucose was inhibited. The accumulation of the 1-phosphate in this case cannot as yet be explained.

The second compound for consideration is lactic acid, which accumulated in normal blood incubates, but not in incubates containing trypanosomes. Moreover, when glucose metabolism was blocked by iodoacetic acid, lactic acid initially present disappeared during incubation. These observations suggest that indigenous lactic acid can be converted to pyruvic acid by the parasites. Further evidence of this possibility is provided by the observed accumulation of more than the theoretical two molecules of pyruvic acid in the presence of stilbamidine.

Summary

1. Investigations of the oxygen utilization of suspensions of *T. evansi* show that this trypanosome metabolizes glucose mainly to pyruvic acid, though a small quantity of this end-product is probably further utilized by decarboxylation.

2. Analysis of trypanosome suspensions for phosphorylated intermediates in the presence of specific inhibitors shows that the intermediate metabolism of the parasites follows the typical Embden-Meyerhof-Parnas scheme characteristic of yeast and muscle metabolism.

3. At least some of the nutritional factors required in this metabolism may be obtained from the plasma of the host.

4. Trypanosomes appear to be able to utilize the lactic acid in the host blood, probably by conversion to pyruvic acid.

5. Observation of the effects of typical trypanocides suggests that trivalent arsenicals inhibit the hexokinase reaction in trypanosomes ; that pentavalent arsenicals are inactive unless reduced *in vivo* to the trivalent state ; that straight chain diamidines (undecane diamidine) partially inhibit the dehydrogenase system on which oxygen transport depends in trypanosomes ; and that aromatic diamidines (stilbamidine) probably inhibit the decarboxylation of pyruvic acid.

The author is indebted to Messrs. P. A. Hankin and R. W. Neville for valuable technical assistance.

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SULPHETRONE*: A CHEMOTHERAPEUTIC AGENT FOR TUBERCULOSIS: PHARMACOLOGY AND CHEMOTHERAPY

BY

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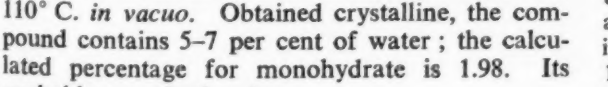
The discovery and evaluation of the chemotherapeutic activity of diaminodiphenylsulphone in these laboratories (Buttle, Stephenson, Smith, Dewing, and Foster, 1937) inaugurated a search which has gone on ever since for a drug which combined the activity of the sulphone with freedom from toxicity to the host. A number of derivatives proved to be active antibacterial agents and were subjected to a comprehensive pharmacological study. The sulphone derivatives include in their range high antibacterial activities to streptococci, pneumococci, and *M. tuberculosis*.

One derivative, 4 : 4′-bis(γ-phenyl-*n*-propylamino) diphenylsulphone-tetrasodium sulphonate, given the registered name "sulphetrone," attracted attention by reason of its freedom from toxicity and its high antibacterial activity. A pharmacological and therapeutic study of this derivative has been made and details are given of the structure, chemical and physical properties, pharmacology, experimental therapy, and possible clinical uses of the drug.

CHEMISTRY

Preparation and properties of sulphetrone

The chemical preparation has been described by Gray and Henry (1936), and by Buttle, Dewing, Foster, Gray, Smith, and Stephenson (1938). The final stage is precipitation with alcohol which yields an amorphous material containing, when air-dried, 5–7 per cent water which it loses when heated to 110° C. *in vacuo*. Obtained crystalline, the compound contains 5–7 per cent of water; the calculated percentage for monohydrate is 1.98. Its probable constitution is:



Molecular weight = 892.5

Typical migrating boundaries may be obtained by electrophoresis, and analysis by this means has enabled Mr. P. A. Charlwood, of these laboratories, to say that the drug as prepared is not homogeneous but consists of a major component of some 94 per cent and a minor component of 6 per cent.

The compound is insoluble in alcohol and other organic solvents, but is exceedingly soluble in cold water to give a syrup. Twenty and forty per cent (w/v) solutions are stable when neutral or slightly alkaline and may be autoclaved. Boiling with normal acid yields a brown coloured complex of high molecular weight and only by more drastic hydrolysis is it possible to recover a small proportion of the whole as diaminodiphenylsulphone.

Estimation of sulphetrone

Sulphetrone was estimated in blood, urine, cerebrospinal fluid and tissues by diazotization and coupling to N-(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939) and estimating the pigment colorimetrically or, preferably, absorptiometrically. Although the facility with which derivatives of diaminodiphenylsulphone are adsorbed by undenatured protein is not shared by sulphetrone, admixture with precipitated proteins is intimate, and therefore the conditions governing the optimum recovery of drug were determined experimentally. When the following conditions are rigidly adhered to, 90 per cent recoveries of drug are obtained; the over-all dilution of 1 in 15 and the concentration of acid are critical: 0.5 c.c. blood, or other body fluid, is added to 5 c.c. *N*/1 HCl and mixed well; 2.0 c.c. 12 per cent (w/v) trichloroacetic acid are added, and the well-mixed solution filtered immediately through

* The development of sulphetrone is part of the programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain, Limited.

a No. 5 Whatman paper and repassed until brilliant; 3 c.c. of filtrate are mixed with 0.05 c.c. 0.3 per cent fresh (weekly) sodium nitrite and left 3 min.; 0.05 c.c. 1.5 per cent ammonium sulphamate is now added and the solution left 2 min.; finally, 0.05 c.c. 0.1 per cent N-(1-naphthyl)-ethylenediamine hydrochloride is added and mixed well. The colour should be allowed to develop for 30 min. before being read colorimetrically or absorptiometrically with a Wratten 61 filter.

The visible absorption spectra of the naphthylethylenediamine derivatives of diaminodiphenylsulphone and sulphetrone are shown in Fig. 1. The colour intensity of the sulphetrone-dye complex is much less than that of the parent substance, although the optimum conditions for colour production were found experimentally and the concentrations were adjusted to be approximately equivalent; this suggests that sulphetrone may not be readily hydrolysed, a suggestion borne out by the pharmacological evidence. The ultra-violet absorption spectra of the derivative and the parent compound are included in Fig. 1.

All estimations of sulphetrone are given in terms of the anhydrous compound.

By collaboration with The Tintometer, Ltd., a standard Lovibond colour disc is available for the rapid reading of blood and body-fluid estimates falling within the range of 0 to 9 mg. per 100 c.c.

Toxicity

Acute toxicity

A 10 per cent (w/v) solution of sulphetrone is isotonic with 0.91 per cent sodium chloride, and hypertonic solutions up to 60 per cent (w/v) are readily obtained. The need to administer large volumes of these grossly hypertonic solutions, together with poor absorption from the gut and low intrinsic toxicity, make the determination of acute toxicity in precise figures of questionable significance.

The results of an estimate of oral toxicity are shown in Table I. All mice receiving 2.0 g. per kg. as 60 per cent solution, and those which died after 1.75 and 1.50 g. per kg., died quickly, after collapse and circulatory failure. *Post mortem* there was gross dehydration and collapse of major blood vessels, the stomach and gut were distended and the contents contained blood. The opinion was formed that the hypertonicity of the solutions made a major contribution to the death of the animals. For example, the blood concentrations at death after the three higher doses appear to be related to hypertonicity and time of death, and are in marked contrast to the concentration found at the fifth hour in the fourth group.

TABLE I

Toxicity for mice of sulphetrone given orally in solution

Dose g. per kg.	Dead/Total	Time of death in hours	Mean blood concentration at death; mg. per 100 c.c. ± S.D.
2.00	20/20	0.75	960 ± 330
1.75	12/20	1.50	2700 ± 874
1.50	4/20	within 5.00	240 ± 19.9
1.25	0/20	—	Mean blood level of all survivors at 5 hours: 14 ± 2

Intravenous administration of a 20 per cent (w/v) solution caused deaths in groups of mice at

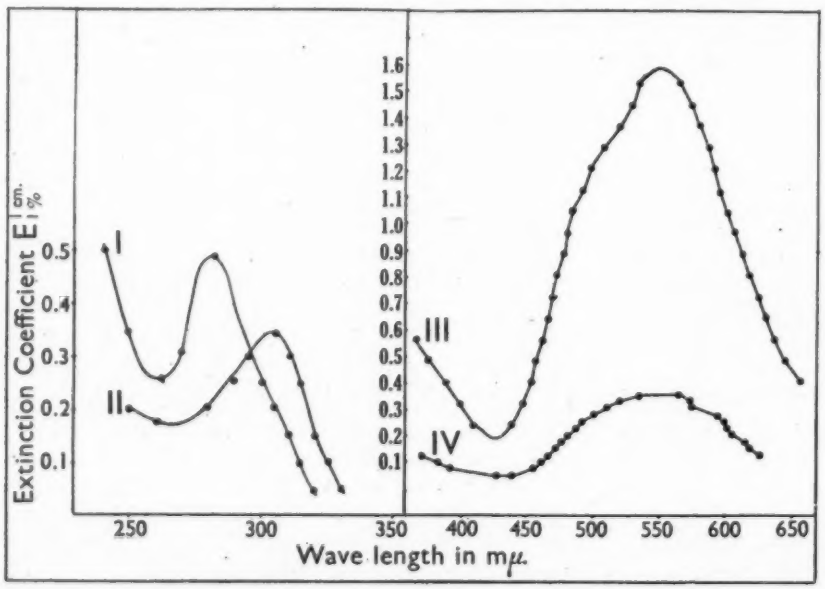


FIG. 1.—Spectrometic measurements of diaminodiphenylsulphone I and sulphetrone II in the ultra-violet, and of diaminodiphenyl-sulphone-naphthylethylenediamine dye III and sulphetrone-naphthylethylenediamine dye IV in the visible wavelength. Ordinates, extinction coefficient, $E^{1cm}_{1\%}$. Abscissae, wavelength, in mμ. I, 1.018 mg. per 100 c.c. *N*/10 HCl; II, 1.022 mg. in 100 c.c. H_2O; III, 0.25 mg. per 100 c.c. *N*/1 HCl; IV, 0.8 mg. per 100 c.c. *N*/1 HCl.

doses of 2.5, 2.75, and 3 g./kg. but not at 1.5 g./kg., at which dose level the mean blood concentration was 650±160 mg. per 100 c.c. Most deaths took place within one minute. The mice became cyanosed, involuntary spasms were overtaken by epileptiform convulsions which passed to extensor hind-limb paralysis; dyspnoea was followed by apnoea. There were a number of late deaths (1–2½ hours). The results are set out in Table II. Computed by the method of Bliss (1938) the LD50 is 2.7 g. with a range of 2.4 to 3 g. per kg.

TABLE II

Toxicity for mice of sulphetrone given intravenously in solution

Dose g./kg.	Dead/ Total	Time of death	Mean blood concentration at death; mg. per 100 c.c. ± S.D.
2.5	6/20	4 in 1 min. 2 in 2.5 hrs.	1000 ± 200
2.75	14/20	10 in 1 min. 4 in 1 hour	1150 ± 240
3.00	14/20	14 in 1 min.	1400 ± 400

A dog was given 1 g. sulphetrone per kg. intravenously in 40 per cent (w/v) solution (a total of 10 g.); the resulting blood concentrations were 61.8 mg. per 100 c.c. at 15 min., 14 mg. at 1 hour, 18 mg. at 2 hours, and 6.8 mg. at 24 hours. It will be seen that urinary clearance was rapid. Apart from lethargy and unsteadiness of gait, no symptoms were observed until after one hour, when the animal had a rigor with tachycardia lasting for 15 min. Two hours after the injection the animal was in high spirits. Intraperitoneal injection of 2 g. per kg. in 40 per cent (w/v) solution was followed by essentially the same symptoms and resulted in a blood level of 96.4 mg. per 100 c.c. at 15 min., 18 mg. at 1 hour, and 2 mg. at 24 hours.

After 2 g. per kg. intravenously the dog collapsed for a brief period 20 min. after the injection; 20 min. later there was a rigor and vomiting and a period of tachycardia for 15 min. The animal was lethargic but recovered, and appeared normal in 2 hours. Blood levels were 160 mg. per 100 c.c. at 15 min., 38 mg. at one hour, and 2.5 mg. at 24 hours.

Experiments on the effect on alkali reserve, described later, suggest that some but not all of the immediate effects after large intravenous doses may be associated with increased plasma alkali. However, since blood concentrations of the simple sulphonamides of half this magnitude cause severe symptoms and death in dogs, it is apparent that sulphetrone has little acute toxicity in the sense in which this term is normally used.

Chronic toxicity

Four adult rabbits of mixed sexes weighing 2.5–3 kg. were kept in metabolism cages for 40 days, during which period they received a diet containing 4 per cent sulphetrone and consisting of bran, sugar-beet pulp, and water. Two additional rabbits were fed upon the diet without drug.

A record was made of the drug intake, together with daily estimations of the concentrations of drug in blood, urine, and faeces. Estimations were made of reticulocytes, erythrocytes, total

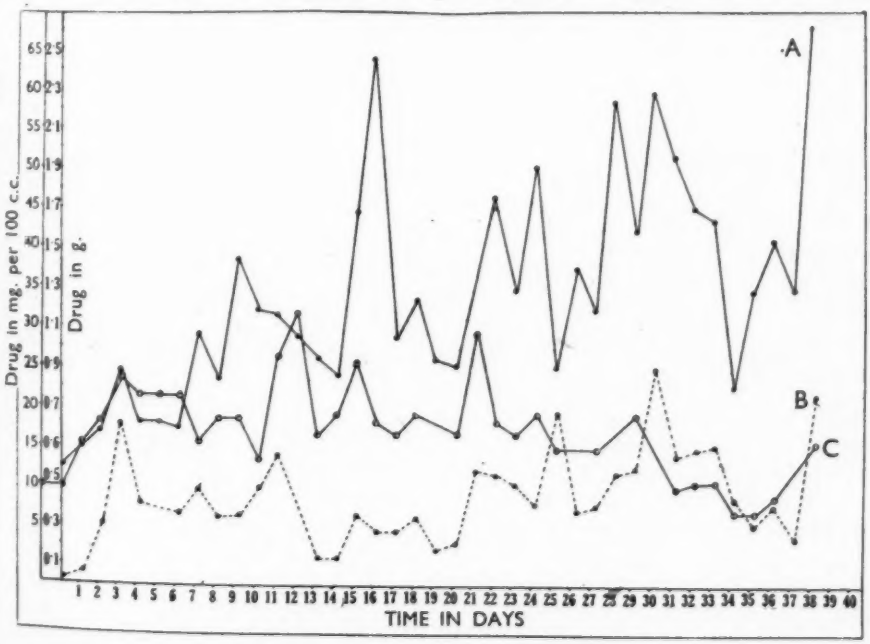


FIG. 2.—Sulphetrone drug-balance experiments in a group of four rabbits receiving 4 per cent of drug in the diet. A, average daily drug in urine and B, average daily drug in faeces, the ordinates being drug in grams. C, average daily blood concentration, the ordinates being drug in mg. per 100 c.c. Equilibrium is established after 3 or 4 days. Between days 6 and 20, about one-third of the drug is unabsorbed; subsequently this figure drops to about one-quarter.

B

white-cell count, and haemoglobin concentration. Although no abnormalities were detected in the white-cell count, progressive hypochromic anaemias were observed in 3 out of 4 rabbits. In one rabbit this was the cause of death at the 15th day. In Fig. 2 are graphed the mean amounts of drug in urine (A) and faeces (B) and the blood level (C) in mg. per 100 c.c. Equilibrium was achieved after about three days. At first about 1/3, but later only about 1/4, was unabsorbed, After equilibrium had been achieved the blood level averaged 18.6 mg. per 100 c.c. ± S.D. 6.0.

Treatment of hypochromic anaemia in rabbits

During the first experiment on rabbits no abnormal excretion of blood pigments was observed. Occasional (8) estimates of methaemoglobin by Zeiss pocket spectroscope were negative. Although the urine was darker in colour than usual no abnormal urobilin excretion was observed. Soon after the administration of the drug there was a high colour index, but this shortly fell, and the anaemia became hypochromic in type. Reduced haematopoiesis rather than abnormal degradation was suspected.

In a second experiment four adult rabbits, two male and two female, of 2–2.5 kg. weight, were fed for 25 days with a diet of bran, sugar-beet pulp, water, and 4 per cent of sulphetrone. In addition to estimates of drug balance, daily estimates were made of reticulocytes, erythrocytes, haemoglobin, and urobilin. Estimates of total urinary porphyrins excreted and of methaemoglobin were also made. The observations are summarized in Fig. 3. A slight though significant increase in urobilin occurs at once and is continued throughout the experiment; reticulocytosis indicates an attempt to make good the deficiency of cells, but an increase in colour index shows this to fail. This state corresponds to a slight but persistent haemolytic anaemia. At this stage a precipitous anaemia with a raised colour index intervenes; this responds to iron, peroral or parenteral, but only to a limited degree.

Absorption of the iron salt of sulphetrone

The fact that iron by mouth stimulated haematopoiesis suggested a direct interference by sulphetrone with iron metabolism. The iron salt was prepared by Mr. W. H. Gray of the Wellcome Laboratories for Research in Tropical Medicine, and its absorption after oral doses was compared with sulphetrone. Given orally the iron complex is completely unabsorbed; parenterally it is distributed like sulphetrone. This finding taken with the evidence of haematopoiesis after oral iron (Fig. 3) indicates that sulphetrone combines with alimentary iron and prevents its absorption.

Modification of intestinal flora by sulphetrone

At this stage, as a result of comparative tests with mice (Brownlee and Tonkin, 1941) the marked ability of sulphetrone to modify the intestinal flora was appreciated. The extent to which many animals, and particularly ruminants, rely on biosynthesis for growth factors, some of which are vitamins, suggested a third experiment with the same diet but containing 10 per cent of dried yeast. Four adult rabbits, male and female, weighing 2.5–3.5 kg. were kept in metabolism cages for 39 days on the modified diet containing 10 per cent dried yeast. The drug balance figures were essentially similar to those of the first experiment. The animals maintained good condition and put on weight; at autopsy on the 45th day, no gross changes were seen in any of them. Histology of spleen, kidney, and red bone-marrow showed a normal picture. Slight cloudy swelling of some peripheral liver cells was the only abnormality observed. The results of blood examination (Fig. 4) justify the conclusion that the gross anaemia previously observed arose directly from a lack of an essential metabolite previously furnished largely by biosynthesis which was prevented by unabsorbed sulphetrone residues and was now furnished by dried yeast. When this form of anaemia is excluded hypochromic anaemia attributable to iron lack is still present. On the third day after withdrawal of drug the residual haemolytic anaemia was made good.

Specific toxic effects

It is known that sulphonamide drugs share with certain thioureas the property of causing hyperaemia and hyperplasia of the thyroid gland in some animals and in man, when they are administered at dose levels and for times similar to those in therapeutic use. The hyperplasia is believed

TABLE III

Goitrogenic effect of drugs fed to groups of five littermate rats as 1 per cent of the diet, for 17 days

Groups	Average body weight, g.		Deviation weight per cent	Thyroid hyperaemia	Thyroid weights mg. per 100 g.
	Initial	Final			
Control	47.2	97.5	103	—	10.1
Sulphetrone ..	45.7	92.0	100.5	+	13.6
Sulphadiazine ..	48.0	88.0	83	+	17.6
Sulphaguanidine	55.0	93.8	70	++	20.1

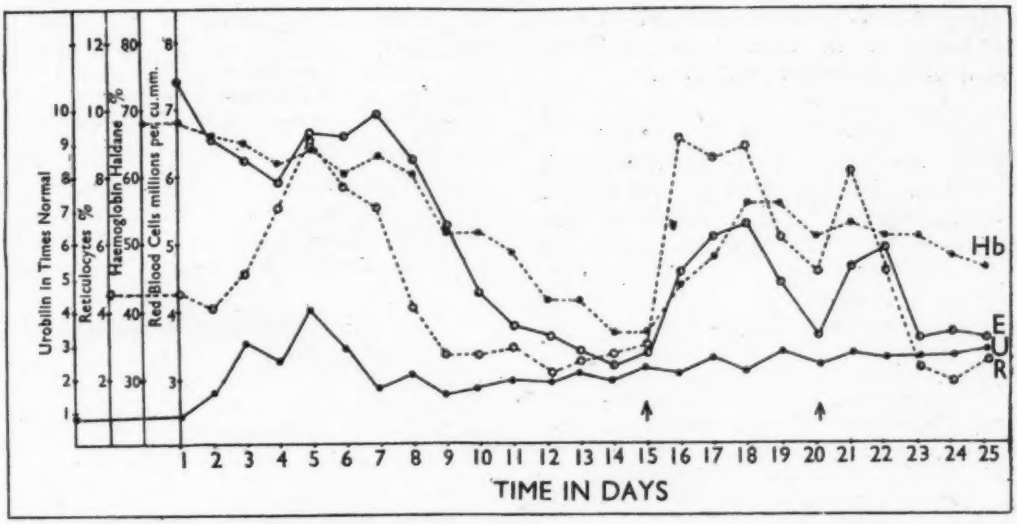


FIG. 3.—Typical response of a rabbit (335 ♂) to a 4 per cent sulphetrone diet. Hb is the haemoglobin estimate (Haldane); E, red blood cells in millions per cu.mm.; R, reticulocytes in per cent of red blood cells; U, spectrometric estimates of urobilin (Watson, 1936) in times normal. At each arrow, 10 mg. of iron was given by mouth, in the form of iron ammonium chelidamate.

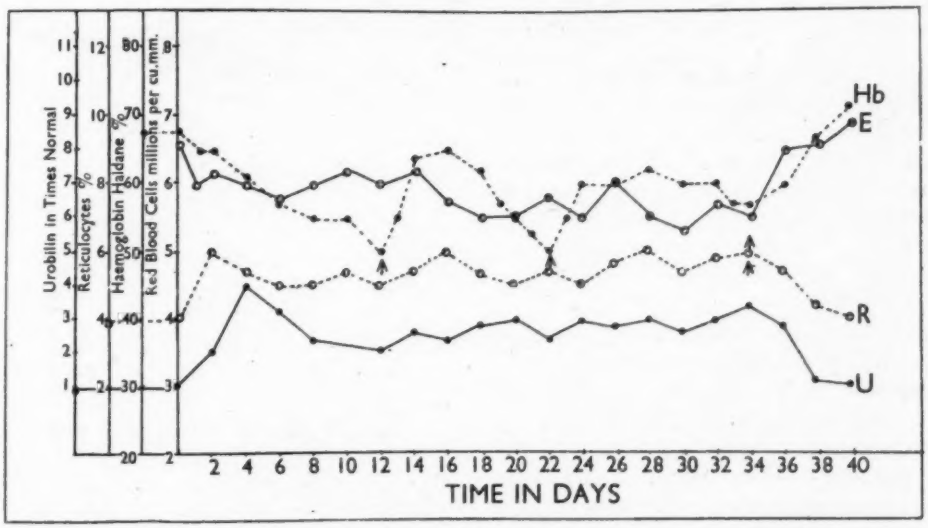


FIG. 4.—Typical response of a rabbit (336 ♂) to a 4 per cent sulphetrone diet, but containing in addition 10 per cent of dried yeast. Hb, is the haemoglobin estimate (Haldane); E, red blood cells in millions per cu.mm.; R, reticulocytes in per cent of red blood cells; U, urobilin by spectrometric estimates of urobilin (Watson, 1936) in times normal. At each single arrow, 10 mg. of iron, in the form of iron ammonium chelidamate was given by mouth. At the double arrow drug was withdrawn.

(Astwood, Sullivan, Bissell, and Tyslowitz, 1943) to result from a hypothyroidism produced by failure to synthesize thyroid hormone. The goitrogenic activity decreases from sulphadiazine, sulphapyridine, sulphathiazole, sulphaguanidine, sulphanilylurea, sulphanilamide to sulphasuxidine (MacKenzie and MacKenzie, 1943).

Sulphetrone was tested for this specific toxic effect together with sulphadiazine and sulphaguanidine in litter-mate groups of five immature female rats of Wistar strain fed on a powdered diet containing 1 per cent of the drugs for a period of 17 days. The thyroids were removed, weighed, and studied histologically. The results summarized

in Table III show a decrease in goitrogenic activity from sulphaguanidine and sulphadiazine to sulphetrone, which shows a slight toxic effect.

Effect on normal growth of rats

The effect of feeding a synthetic diet containing 1 per cent of sulphetrone and 0.01 per cent of iron to litter-mate groups of rats for 78 days is summarized in Table IV. Both the female group and

TABLE IV

The effect on the growth-rate, and thyroid weights, of a 1 per cent sulphetrone diet fed to two litter-mate groups, male and female, of rats, for 78 days. Male and female control groups are included

Rats Litter-mate groups of eight	Average weight in g.		Thyroid weights mg. per 100 g. mean ± S.D.
	Initial	After 78 days	
Drug 1% ♂	34.0	180.6	9.2 ± 0.8
Controls ♂ ..	33.9	192.1	8.9 ± 0.7
Drug 1% ♀	30.9	138.0	11.5 ± 1.1
Controls ♀ ..	32.8	149.1	9.9 ± 0.8

the male group were retarded in growth for the first two weeks, but thereafter increased in weight by normal increments so that graphs of the growth-rates followed parallel courses. The weight increases of the thyroid glands are not significant in comparison with those produced by treatment with sulphonamides.

Effect on alkali reserve

The evidence of acute and chronic toxicity, together with the spectrometric evidence of the difference between the dye-coupled products of sulphetrone and of diaminodiphenylsulphone, shows that sulphetrone is not hydrolysed to diaminodiphenylsulphone in the body. Since large amounts of the drug pass through the blood stream, the probable effects of hydrolysis of one or more of the sodium sulphonate radicles on the alkali reserve were investigated. The results, which were unexpected, are discussed later.

Groups of adult rabbits of mixed sex were maintained on a diet of bran and oats containing 50 per cent of water for one week to stabilize their metabolism, and were then given a single oral or parenteral dose of 1 g. sulphetrone per kg. as a 50 per cent (w/v) solution. At intervals blood was withdrawn from the heart into an oxalated syringe, immediately centrifuged under paraffin, and the CO_2 capacity determined by the volumetric method of Van Slyke. The results (Table V) show a rise in

TABLE V

Plasma CO_2—combining capacity, in volumes per cent, after oral and intraperitoneal doses of 1 g. per kg. sulphetrone in rabbits

Rabbit	CO_2 capacity of plasma (vols. %) after						Mean blood concentration mg. per 100 c.c. ± S.D.
	0 hr.	1 hr.	2 hr.	4 hr.	6 hr.	24 hr.	
1 intraperitoneal	32.9	39.6	45.4	52.1	44.3	—	75 ± 25
2 intraperitoneal	32.0	40.6	42.5	42.5	47.4	41.3	40 ± 32
3 intraperitoneal	40.6	39.0	36.0	40.3	40.6	—	—
4 oral	40.6	39.0	36.0	40.3	40.6	—	6.0 ± 6
5 oral	38.1	41.0	47.0	48.5	40.3	—	2.5 ± 1

the alkali reserve after an oral dose of sulphetrone in one of two experiments, and after an intraperitoneal injection in two of three experiments.

The effect of parenterally administered drug is the same in the dog (Table VI) and it is interesting

TABLE VI

Plasma CO_2—combining capacity, in volumes per cent, after parenteral sulphetrone or sodium bicarbonate in dogs

Dog	CO_2 capacity of plasma (vols. %) after						Mean blood concentration mg. per 100 c.c. ± S.D.
	0 hr.	1 hr.	2 hr.	4 hr.	6 hr.	24 hr.	
A. Sulphetrone 2g./kg.I.P.	53.1	—	56.7	57.1	57.1	57.3	17.7 ± 12
B. Sulphetrone 1g./kg.I.V.	54.6	62.0	68.5	52.5	61.5	65.0	8.5 ± 4
C. Sulphetrone 1g./kg.I.V.	43.5	46.5	49.0	51.0	52.0	—	22.0 ± 16
D. Sodium bicarbonate 94 mg./kg. I.V.	40.9	48.9	50.4	48.1	—	—	—
E Sodium bicarbonate 188 mg./kg. I.V.	43.6	55.1	52.0	53.5	51.7	—	—

that the effect of 1 g. sulphetrone per kg. is approximately matched by 94 mg. sodium bicarbonate per kg. injected intravenously. This equivalence corresponds to the sodium of one of the four sodium sulphonate radicals being set free as sodium hydroxide. Dogs injected intravenously with this dose of sodium bicarbonate are subdued and

lethargic for about 30 minutes but show no other signs of toxicity.

The alkali reserve after repeated doses of sulphetrone

For the purpose of a "chronic" test a total daily dose of 0.5 g. sulphetrone per kg. was given orally in 2 portions as a 5 per cent (w/v) solution. Five adult rabbits fed on a diet of bran, sugar-beet pulp, and water were divided into groups, one of which received drug and the other water for a period of 7 days. Eighteen hours after the last treatment the CO_2 capacity of the plasma was estimated. Treatment was now resumed so that rabbits which had been given water now received sulphetrone and vice versa. The plasma CO_2 capacity, together with the blood concentrations of sulphetrone at the end of four weekly periods, are given in Table VII, which shows that when repeated doses are given from day to day equilibrium is achieved.

TABLE VII

The CO_2 capacity of the plasma of rabbits, as volumes per cent, after repeated doses of sulphetrone in a "cross-over" test

Rabbits	CO_2 capacity of plasma as volumes per cent after		Blood concentrations of drug, mg. per 100 c.c.
	Water 5 c.c./kg. twice daily	Sulphetrone 5 c.c. of 5% twice daily	
4	55.0	33.0	10.7
5	28.5	44.5	6.0
6	46.0	48.0	8.0
7	47.0	35.0	8.8
8	41.5	59.0	9.0
9	46.0	51.0	5.2
10	37.0	39.5	8.8
11	50.0	—	—
12	53.0	51.5	3.0
13	49.5	41.0	9.0
Mean value ± S.D.	45.3 ± 8.1	44.7 ± 8.4	7.6 ± 2.5

Absorption and Excretion

When sulphetrone in solution was given by mouth to groups of mice, the blood concentration–time curves were about the same for large and small doses. Typical results are given in Fig. 5, curves for intravenous and intraperitoneal injections being also included; curves for oral doses of diaminodiphenylsulphone and sulphanilamide are included for comparison.

A closer relation between large and small doses is seen in dogs after single doses given orally.

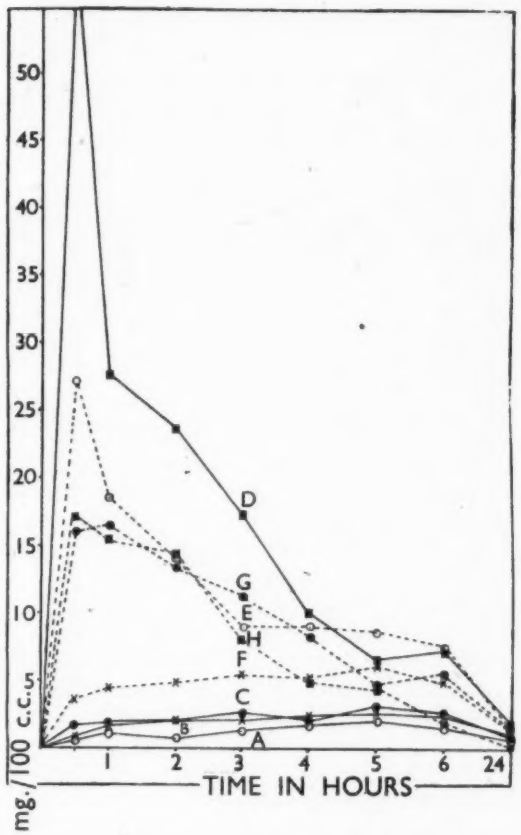


FIG. 5.—Blood concentration-time curves after sulphetrone, diaminodiphenylsulphone and sulphanilamide. Each curve is the mean of determinations on five fasting mice. A, sulphetrone, 0.1 g./kg. by mouth. B, same, 0.5 g./kg. C, same, 1.0 g./kg. D, same, 0.1 g./kg. as 40 per cent solution intravenously. E, same, 0.1 g./kg. as 40 per cent solution intraperitoneally. F, diaminodiphenylsulphone, 0.1 g./kg. suspended in acacia by mouth. G, same, 0.25 g./kg. H, sulphanilamide, 0.1 g./kg. suspended in acacia by mouth.

Blood and urine concentrations after intravenous doses are also included in Table VIII.

The total quantity excreted in the urine is given in per cent of the amount administered. It is interesting that although drug given intravenously is cleared from the blood stream rapidly and excreted almost completely in the urine in 24 hours, only 75 per cent of an oral dose is excreted in the same time. Evidence is given later that poor absorption from the bowel, and in particular from the large intestine, may account for the remainder.

Blood concentration–time curves obtained in a dog after an oral dose, and after the same dose given in three divided portions at three-hourly in-

TABLE VIII

ABSORPTION AND EXCRETION OF SULPHETRONE IN DOGS

Dog	Dose g./kg.	Blood concentrations, in mg. per 100 c.c. blood, at hours:							Excreted in per cent of total dose at hours:			
		0.25	1	2	3	4	6	24	2	3	6	24
Terrier 1	0.1	1.4	2.8	4.1	3.8	2.8	1.2	0.1	20.4	50.1	62.7	76.0
	0.2	0.9	3.3	—	3.2	1.6	1.4	0.3	19.4	39.2	49.6	67.4
	0.5	1.8	7.0	7.2	4.5	4.2	2.2	0.0	23.2	29.8	64.4	71.2
	1.0	1.6	7.2	9.4	7.2	5.2	4.1	0.2	24.3	49.7	59.4	73.9
Greyhound 2	0.2	0.4	2.8	4.6	4.5	3.9	2.7	0.1	19.8	37.4	49.2	67.8
	0.5	0.2	6.2	6.3	4.8	4.1	1.2	0.2	18.2	39.4	62.4	78.4
Mongrel 3	0.5	1.0	4.2	3.9	2.6	1.9	2.1	0.4	17.2	28.4	42.6	66.4
	1.0	0.2	4.7	4.9	6.2	5.3	4.0	0.2	24.6	33.2	47.6	75.2
Terrier 1*	0.1	12.1	4.4	3.2	1.8	1.4	1.0	0.6	69.7	69.9	82.6	98.6
	0.2	20.5	6.0	4.5	3.4	2.4	1.8	0.8	64.2	68.4	78.4	96.4

* Intravenously

tervals (Fig. 6), suggested that absorption took place high in the alimentary tract. An experiment in which the drug was injected first into the large and then into the small intestine supports the view that absorption is largely confined to the small intestine (Fig. 7).

Renal clearance in the rabbit

The essential preliminary to a study of the elimination of sulphetrone from the body is a knowledge of its mode of excretion by the kidney. In a study in the dog, Marshall, Kendall, and Cutting (1937) found the clearance of sulphanilamide to be 20 to 30 per cent of a simultaneously determined creatinine clearance. In an experiment in which a group of three rabbits was used the clearance of sulphetrone was 58 per cent that of creatinine, or two to three times as fast as sulphanilamide. The result was the same whether the plasma concentration of the drug was produced by prolonged administration for 40 days or by a single dose. A typical estimate based on single doses is shown in Table IX. Each ratio is a mean of six periods of one experiment, thus Rabbit X, 0.62 ; Y, 0.63 ; and Z, 0.5 ; giving a grand mean of 0.58.

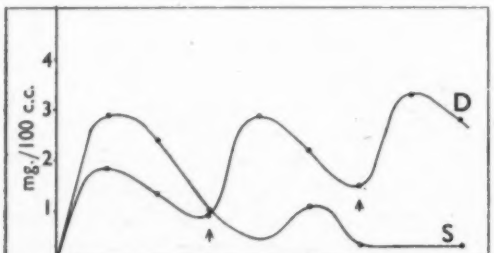


FIG. 6.—Blood concentration-time curves in a dog after sulphetrone by mouth. S, a single dose of 0.2 g. per kg. in 60 per cent solution at first arrow. D, a divided dose of 0.1 g. per kg. given at each arrow.

Renal clearance in the dog

Renal clearance for the dog has been calculated from data, some of which are given in the last

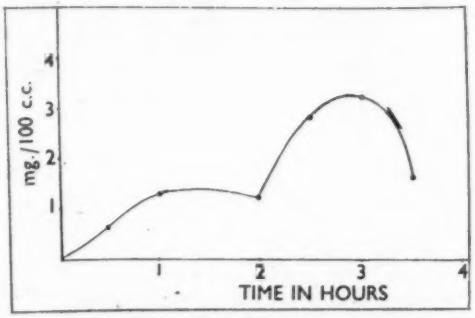


FIG. 7.—Blood concentration-time curves showing absorption from large and small intestines. Dog anaesthetized with pentobarbitone-sodium. Tube passed through oesophagus and pylorus into duodenum, and pylorus tied; a second tube passed through anus into the descending colon, the tube being tied at the anus to prevent leakage and the ileocaecal junction tied. Sulphetrone 0.1 g. per kg. in 40 per cent solution introduced by the oral tube and washed out after two hours; 0.1 g. per kg. then introduced into the duodenum.

TABLE IX

SULPHETRONE AND CREATININE CLEARANCE IN THE RABBIT

The rabbit weighed 2.2 kg. and received 1 g. of creatinine and 3 g. of sulphetrone dissolved in 25 c.c. of water. This quantity represented the third of three volumes of water given at 20 minute intervals

Period	Mins.	Urine A c.c./min.	Creatinine mg./100 c.c. B plasma	Creatinine mg./100 c.c. C urine	Sulphetrone mg./100 c.c. B plasma	Sulphetrone mg./100 c.c. C urine	Clearance $\frac{C \times A}{B}$ Units per min. Creatinine	Clearance $\frac{C \times A}{B}$ Units per min. Sulphetrone	Ratio Sulphetrone / Creatinine
1	27	0.57	9.2	350	4.1	102.0	2.15	1.4	0.65
2	37	0.39	11.6	133	7.5	56.6	4.45	2.94	0.66
3	30	0.25	12.0	160	8.5	59.2	3.35	1.75	0.52
4	23	0.83	11.2	265	8.9	46.7	19.5	4.3	0.22
5	60	0.34	10.1	240	4.0	171.5	8.2	6.44	0.78
6	60	0.37	8.3	270	8.9	232.4	11.5	9.6	0.98

experiment in Table VIII. Six determinations gave a mean clearance ±S.D. of 85.2±14.3 units ; six sulphanilamide clearances on the same animal gave a mean clearance ±S.D. of 17.9±3.1 units. Accepting creatinine clearance as a rate of glomerular filtration it seems that some 40 per cent of sulphetrone is resorbed in the passage of glomerular filtrate along the tubules in the rabbit, while in the dog the quantity resorbed is very slight.

Distribution of sulphetrone in tissue

The penetration of sulphetrone into the tissues, with the exception of brain and cerebrospinal fluid, is rapid and complete. The distribution and concentration is the same whether the drug is given in the diet for prolonged periods or acutely by one intravenous dose (Table X).

The similar tissue concentrations in animals receiving drug by one intravenous dose, and in the diet for 10 weeks, point to the importance of renal clearance as one major controlling factor. In this connection the low concentration in plasma compared with liver and kidney, but not bone-marrow or lung, indicates that the liver also is functioning as an organ of concentration and excretion. The high concentration in bile supports this view. That neither the rate of excretion nor the route are the sole controlling factors may be deduced from Table XI, where the distribution in the tissues of the nephrectomized rabbit is given. In spite of striking increases in the plasma concentration

TABLE X

The penetration of sulphetrone into various tissues in the rabbit and the dog, expressed as mg. per 100 c.c. of fluid and mg. per 100 g. of tissue

Tissue	Rabbit (2 kg.) 1% sulphetrone in diet 10 weeks	Rabbit (1.75 kg.) 100 mg. sulphetrone per kg. intravenously. Estimates at 2 hrs.	Dog (12 kg.) 100 mg. sulphetrone per kg. intravenously. Estimates at 2 hrs.
Blood	3.3	3.8	4.1
Plasma	6.3	7.4	6.7
Corpuscles	0.8	1.2	1.1
C sternal fluid	1.1	0.4	1.5
Bile	80.0	92.0	79.0
Liver	14.0	12.9	11.5
Kidney	13.1	15.3	20.4
Spleen	7.1	6.2	9.8
Lung	4.8	4.1	6.2
Bone-marrow	3.1	2.4	0.8
Striated muscle of thigh	1.6	2.0	6.3
Ileum, empty	8.6	1.6	4.5
Cerebral hemispheres	0.9	0.5	1.9
Vitreous humour	0.8	0.7	0.4
Fat	0.9	1.1	1.2

TABLE XI

The distribution of sulphetrone in the tissues of the nephrectomized rabbit at 2 hours after 100 mg. per kg. intravenously. Kidneys were removed under pentobarbitone-sodium, 30 mg. per kg.

Tissue	Rabbit (1.65 kg.) mg./100 g. or c.c.	Rabbit (1.60 kg.) mg./100 g. or c.c.	Rabbit (1.70 kg.) mg./100 g. or c.c.
1 Hour			
Blood	20.5	22.9	19.5
Plasma	42.6	53.8	35.5
Corpuscles	—	12.4	5.5
2 Hours			
Blood	30.8	—	21.2
Plasma	39.2	43.0	35.0
Corpuscles	—	6.2	4.6
Cisternal fluid	1.3	trace	trace
Bile	136.0	—	170.0
Liver	10.3	15.0	11.9
Spleen	6.5	7.5	8.1
Lung	11.8	18.6	13.8
Bone-marrow femur	11.0	12.5	8.1
Striated muscle of thigh	4.4	3.0	2.6
Ileum, empty	8.8	11.1	8.5
Cerebral hemispheres	1.8	1.2	0.9
Vitreous humour	1.3	—	—

(about five times) little change is reflected in the concentrations in the viscera. Biliary excretion is significantly high and concentration of drug in the ileum indicates that this, too, is an organ of excretion.

Other pharmacological properties

Examined by the usual pharmacological methods, sulphetrone is inert. Thus, a solution of 1 in 1,000 has no action on the isolated guinea-pig uterus or on the rabbit intestine *in vitro* at 37.5° C., or on the frog heart perfused through the vena cava. A cat anaesthetized with phenobarbitone-sodium (30 mg. per kg.) and given 0.2 g. sulphetrone per kg. intravenously showed no demonstrable effect on blood pressure, heart rate, respiratory frequency, or volume.

Experimental Therapy

Antibacterial activity

Our procedure for measuring antibacterial activity of sulphonamide-type compounds *in vitro* consists of observing the effect of exposing constant inocula of selected pathogens to successive dilutions of known concentrations of test and standard drug in media containing 10 per cent of blood. Results of tests to determine the limiting inhibitory concentrations of sulphetrone, promin, and diaminodiphenylsulphone in Wright's broth containing 10 per cent of blood against Gram-positive and Gram-negative organisms, and in Long's medium containing 10 per cent of blood against *M. avium* and *M. tuberculosis* var hominis and var bovis, are summarized in Table XII. Initial drug concentrations of 2.0 g. per litre were made and diluted out in the medium to obtain a series of drug concentrations which decreased by multiples of two. Drug solutions in the test medium were autoclaved, mixed with blood, and inoculated with 0.2 c.c. of a suitably diluted culture. Size of inoculum and conditions of test were such as to demonstrate antibacterial activity under conditions which permitted turbid growth in the control tubes in less than 24 hours. Initial bacterial viable counts in test mixtures were made by roll-tube counts.

TABLE XII

The limiting inhibitory concentrations of diaminodiphenylsulphone, sulphetrone, and promin against different organisms *in vitro*

Organisms		Diaminodiphenylsulphone	Sulphetrone	Promin
Str. pyogenes	CN. 10	2^{-9}	2^{-7}	
D. pneumoniae	CN. 33	2^{-6}	2^{-4}	
S. aureus	CN. 491	2^{-5}	2^{-1}	
S. salivarius	CN. 30	2^{-5}	2^{-1}	
,, ,,	CN. 199	2^{-11}	2^{-9}	
,, ,,	CN. 13	2^{-8}	2^{-6}	
E. typhosa	CN. 512	2^{-4}	2^{-1}	
Sh. sonnei	CN. 188	2^{-5}	2^{-2}	
Sh. dysenteriae	CN. 191	2^{-4}	2^{-4}	
Sh. paradysenteriae	CN. 185	2^{-6}	2^{-4}	
E. coli	CN. 150	2^{-4}	2^{-2}	
V. comma	CN. 249	2^{-6}	2^{-4}	
Cl. perfringens	CN. 1491	2^{-3}	2^{-2}	
Cl. septicum	CN. 368	2^{-8}	2^{-7}	
Cl. novyi	CN. 735	2^{-2}	2^{-1}	
M. avium	CN. 281	2^{-7}	2^{-6}	2^{-5}
,, ,,	CN. 280	2^{-6}	2^{-5}	2^{-4}
M. tuberculosis var hominis				
	CN. 844	2^{-4}	2^{-3}	2^{-2}
	CN. 271 (H37)	2^{-6}	2^{-5}	2^{-4}
	CN. 1877	2^{-6}	2^{-5}	2^{-4}
	CN. 1878	2^{-4}	2^{-3}	2^{-2}
M. tuberculosis var bovis				
	CN. 858	2^{-4}	2^{-3}	2^{-3}
	CN. 868	2^{-6}	2^{-5}	2^{-4}

Reversal by p-*aminobenzoic acid*

Titrations in a semi-synthetic reinforced gelatine-hydrolysate medium containing added cystine and tryptophane, together with the essential growth factors, and inoculated with 1,300 viable organisms of *S. pyogenes* N.10, enabled the concentration of *p*-aminobenzoic acid which just reversed the antibacterial action of sulphetrone to be found. The figure for eight estimates, derived from tenfold dilutions of *p*-aminobenzoic acid from 10^{-3} to 10^{-10}, was $1 : 5{,}000 \pm 500$.

Streptococcus and pneumococcus mouse infections

The chemotherapeutic activity of sulphetrone in mice against a β-haemolytic streptococcus infection (CN.10) and against a Type 1 pneumococcus infection (CN.33) was assessed in a survival test experiment in which groups of 30 mice receiving a diet containing 2 per cent of sulphetrone or of other sulphonamides were inoculated with a number of lethal doses of the test organisms. Drug diet intakes and blood concentrations were measured at 14 days after infection, at which time treatment was terminated. Deaths were recorded daily and the cause of death ascertained by blood culture; survivors were observed for a further seven days. The data from these experiments are recorded in Table XIII for the streptococcus test in which sulphanilamide was used for comparison. Sulphetrone is as good an antistreptococcal drug as sulphanilamide and in terms of a therapeutic factor *F*, obtained by dividing the mean free drug concentration in blood by the mean drug intake, it is more efficient. Rather surprisingly, in view of the efficiency of diaminodiphenylsulphone, sulphetrone has no antipneumococcal activity.

TABLE XIII

Three groups of 30 mice infected with 2 lethal doses (LD100) of *Streptococcus pyogenes* CN.10. All controls were dead within 48 hours and the survivors are shown after 21 days' treatment with 2 per cent drugs in the diet. When the factor *F*, derived from groups of 25 mice on the 14th day, is considered, sulphetrone is the more efficient

Drug 2% in diet	Survivors	Mean drug intake mg.	Mean free drug. Blood conc. in mg. per 100 c.c.	$F = \frac{\text{blood conc.}}{\text{drug intake}}$
Sulphetrone	25/30	69.2 ± 9.5	11.4 ± 3.4	0.161 ± 0.031
Sulphanilamide	27/30	54.6 ± 3.8	18.4 ± 4.2	0.336 ± 0.057

In vivo–in vitro antituberculous test

Before proceeding to a full-scale guinea-pig protection trial it is our custom to exact the following minimum therapeutic requirement from a potential antituberculous drug. Guinea-pigs of 550–600 g. are injected intraperitoneally with 2 g. of the substance suspended in 10 per cent gum acacia and, after two hours or before if symptoms of acute toxicity are seen, they are anaesthetized, the thorax is opened and they are bled aseptically from the heart by Pasteur pipette. By drawing the specimen while the heart still beats, no difficulty is experienced in obtaining more than 3 c.c. of blood, which is stored in a sterile citrated bottle. The blood is diluted in serial increments with equal volumes of 0.5 c.c. of Long's agar contained in previously sterilized and stoppered Lambeth tubes. The tubes are sloped in the usual fashion and sown with 0.01 c.c. of a uniform suspension of *M. tuberculosis* containing 0.5 mg. per c.c. The tubes are incubated at 37.5° C. and inspected at intervals; the inhibition of growth is compared with the inhibition produced by diaminodiphenylsulphone tested in the same way at the same time. Avian strains are read at six days, and bovine and human strains at 21 days. A comparison of sulphetrone and diaminodiphenylsulphone is given in Table XIV.

TABLE XIV

Minimum effective drug blood concentrations in mg. per 100 c.c. blood (from serially diluted blood of a guinea-pig given drug parenterally) which just inhibit strains of mycobacteria

Concentration of drug in guinea-pig blood mg. per 100 c.c.	Diaminodiphenylsulphone	Sulphetrone
M. avium CN. 281	0.14	0.22
M. avium CN. 280	0.14	0.22
M. tuberculosis var bovis CN. 858	75	110
" " " " CN. 868	19	55
M. tuberculosis var hominis CN. 844	75	110
" " " " CN. 271 (H37)	19	55
" " " " CN. 1877	19	110
" " " " CN. 1878	75	110

Discussion

The preparation in these laboratories of diaminodiphenylsulphone and its characterization as a potent antibacterial and chemotherapeutic agent resulted in many attempts here and elsewhere to discover an equally potent but less toxic derivative. With the knowledge of its chemotherapeutic antituberculous effect the search was intensified. Sulphetrone was described in 1938 (Buttle *et al.*, 1938), when its chemotherapeutic antistreptococcal effect was appreciated, but it was not until 1941 that its chemotherapeutic antituberculous action was first realized. While sulphetrone was still under test, preliminary reports of promin (Feldman, Hinshaw, and Moses, 1941), the first diaminodiphenylsulphone derivative to be used in man for the treatment of tuberculosis, appeared. This drug, and the derivatives which have appeared since, diasone and promizole, are very toxic drugs when administered orally and may be given only for short intermittent periods. The fact that sulphetrone proved to be so relatively non-toxic, coupled with the hopes that clinical trials of this kind have always engendered, led us to the conclusion that all publication should be suspended until a mature appreciation could be given; with the publication of clinical reports this can now be done.

The antibacterial activity of sulphetrone approaches closely that of its parent substance, diaminodiphenylsulphone, and suggests that it may prove equally effective in the treatment of experimental tuberculosis in the laboratory animal.

The acute toxicity of sulphetrone when given by mouth is so slight that it cannot with certainty be determined, but the studies are complicated by the soluble nature of the drug which necessitated the administration of grossly hypertonic solutions. On the basis of blood concentrations sulphetrone would seem to be many times less acutely toxic than sulphanilamide. This is in marked contrast to the studies on acute toxicity undertaken with promin, diasone, and promizole.

The fact that sulphetrone is not acetylated, or conjugated in any other way, throws an interesting light on this lack of toxicity. The implication of this finding is that the drug is not hydrolysed to diaminodiphenylsulphone, an inference which is implicit in the lack of acute toxic symptoms observed in the dog with a very high blood level of

sulphetrone. Since in its suggested use, the problem of dosage is to maintain an effective consistent level in blood and other tissue for very long periods of time, we have been interested in the chronic toxicity of long-maintained blood levels.

Chronic toxicity experiments indicate that very large doses can be given by mouth to mice and dogs without producing symptoms or pathological changes in the tissue. With similar doses in rabbits, very interesting haematological changes are seen. First, there is a small but continuous haemolytic anaemia which is marked by a concurrent reticulocytosis. Secondly there is a progressive hypochromic anaemia of iron-lack. This anaemia is due to competition for alimentary iron by sulphetrone which forms an insoluble iron salt, which is not absorbed. It may be prevented or cured by the administration of iron, parenterally or orally.

The third anaemia which is seen is slower to appear but is then precipitous ; it is of nutritional origin and may be prevented or cured by the administration of dried yeast. Its probable cause is the limitation and alteration of the bacterial flora of the gut by the concentration of sulphetrone which is present.

A specific toxic effect which sulphetrone shares with all sulphonamides is the property of causing hyperaemia and hyperplasia of the thyroid gland after courses of treatment similar to those in therapeutic use. The hyperplasia is believed to result from hypothyroidism owing to failure to synthesize thyroid hormone.

Experiments in groups of rats maintained on a diet containing 1 per cent of the drugs for 17 days showed sulphetrone to be the least, and sulphaguanidine the most, toxic, while sulphadiazine was intermediate in this respect.

In a further study of chronic toxicity, it was observed that 1 per cent concentrations of drug in the diet of groups of rats retarded normal growth for an initial period of 14 days, but thereafter increases in weight followed the curve of normal increments during a 78-day period of observation. After this long period of treatment, the increases in the weight of the thyroids were not significantly greater than those in the control groups.

The lack of acute toxicity and the low chronic toxicity, with complete freedom from the toxic sequelae seen after administration of diaminodiphenylsulphone, make it clear that sulphetrone is not degraded to diaminodiphenylsulphone in the body. However, the possible hydrolysis of one or more of the four sulphonated side chains could not be overlooked and alkali reserve experiments were made to study this point. Simple hydrolysis resulting in the liberation of one or more molecules of sodium acid sulphate seemed the most likely event. Experiments in rabbits receiving oral and parenteral sulphetrone and experiments in dogs with the drug administered parenterally showed, however, that alkali was liberated in the blood stream, resulting in consistent increases in alkali reserve. Comparison with parenteral sodium bicarbonate makes it possible to say that the increase corresponds to one molecule of sodium hydroxide being liberated from each molecule of sulphetrone. A theoretical equation, involving the intervention of one molecule of water for the condensation of two molecules of sulphetrone through an $-SO_2$-O-SO_2- linkage with the liberation of two molecules of sodium hydroxide, may be proposed. $2R.SO_3Na + H_2O = R.SO_2OSO_2R + 2NaOH$. Experimental evidence in man points to the existence of a complex condensation product of this kind. When chronic tests were made on groups of rabbits on a "cross-over test" basis, no significant increases in alkali reserve were recorded eighteen hours after the last dose, at which time the average blood levels were 7.0 mg. per 100 c.c. It seems that under these conditions the normal animal is able to re-establish an equilibrium in its plasma alkali balance.

Reviewing all the evidence of toxicity in the experiments described, it seems safe to say that when given by mouth not only is sulphetrone the least toxic of the sulphones, but it is also less toxic than any of the sulphonamide drugs.

Experiments in mice and dogs in which single small or large doses are given show that after a certain point is reached large increases in dose do not result in either a higher blood concentration or increased absorption. This is not an unusual finding with sulphonamide drugs, but it seems to occur with sulphetrone at a lower level of dosage. The rabbit is anomalous in that increase in dosage increases the absorption and blood concentration of sulphetrone. The same total amount of drug given in divided doses at intervals of a few hours results in a higher blood concentration than when given as a single dose (dog). The explanation seems to be that this very soluble drug is slowly absorbed, and mainly from the small intestine. In addition, the rate of excretion of sulphetrone by the kidney is very fast. In rabbits the clearance of sulphetrone was 58 per cent that of creatinine or two to three times as fast as sulphanilamide, while the clearance in the dog was five times as fast as sulphanilamide. If creatinine clearance is accepted as a measure of the rate of glomerular filtration it seems that in the rabbit some 40 per

cent of sulphetrone is resorbed in the passage of glomerular filtrate along the tubules while in the dog the quantity resorbed is very slight. Some part, therefore, of the higher blood concentrations found in the rabbit is contributed by slower clearance.

Sulphetrone penetrates all tissues with extreme rapidity, with the exception of brain, but it enters the cerebrospinal fluid rather more slowly than do other sulphonamides.

The tissues of animals receiving the drug intravenously show similar concentrations to those receiving the drug in the diet for 10 weeks. In the normal animal concentration of drug in the liver, kidney, and spleen is always greater than in the plasma. The relation between absorbed drug, drug in transport, and drug in kidney clearance does not appear to be a simple one, and there appears to be an additional factor limiting drug concentrations in tissue such as liver, spleen, and kidney. The plasma levels in the nephrectomized rabbit make an interesting comment on this observation since they are many times the plasma level in the normal animal and many times the level in the other tissues. In both normal and nephrectomized animals the concentrations in bile are very high ; in the normal they are some 12 times the plasma levels and may be considered as drug in transport, thus making a significant contribution to the clearance of the drug. This view receives support from the fact of the slight decrease in the plasma concentrations in the nephrectomized rabbits between the first and second hours ; the average fall in plasma concentration is some 5 mg. per 100 c.c. which, in a rabbit of 1.7 kg., containing by our records 200 c.c. of plasma, corresponds to a total in bile of 10 mg. of sulphetrone. The recorded bile flow of these anaesthetized rabbits averages 10 c.c per hour, which is equivalent to a sulphetrone loss of 15 mg. It may therefore be argued that in the nephrectomized animal sulphetrone reaches equilibrium within one hour, and the subsequent fall in plasma concentration is due to biliary excretion and not to metabolic destruction.

Sulphetrone has no action on smooth muscle, heart, blood pressure, or respiration in concentrations usual in pharmacological studies.

In considering the data provided by the antibacterial activity of sulphetrone *in vitro* little significance is to be attached to the absolute values recorded for effective inhibitory concentrations. On the other hand, the comparative values for diaminodiphenylsulphone, sulphetrone, and promin are of high significance. In the many comparisons of this kind which have been made in these laboratories the inhibitory concentrations of sulphetrone against mycobacterial strains have always been found to be lower than those observed with promin, and this although, weight for weight, promin contains more diaminodiphenylsulphone. The implication is that the potential cinnamylidene linkages of sulphetrone contribute a significant antibacterial function. No support for this claim can be derived from reversal experiments with *para*-aminobenzoic acid, which reverses the antibacterial rates of sulphetrone at a concentration of 1 : 5,000.

It is surprising, in view of the known efficiency of diaminodiphenylsulphone in pneumococcus infections in mice, that sulphetrone is inactive ; however, in streptococcus infections in mice, sulphetrone is more effective than sulphanilamide.

We have introduced the *in vivo–in vitro* antituberculous test in order to bridge the gap in the difficult transition between antituberculous *in vitro* tests and animal protection experiments with strains of mycobacteria. Whenever the drug can be estimated, and it is seldom that it cannot, either chemically or microbiologically, it is possible to express the inhibition of the organism in a quantitative fashion in terms of a standard such as diaminodiphenylsulphone. When the drug cannot be estimated, the method depends on assessing the inhibition of the organisms produced by the quantity of drug existing in the blood after a dose sufficient to produce toxic signs in the animal, and therefore at an optimum level.

An assessment of the efficiency of sulphetrone in the treatment of experimental infections with strains of *M. tuberculosis* in the guinea-pig is the subject of a separate communication. A study has also been made of the toxicity of the drug when used in man. Certain advantages and disadvantages of sulphetrone as a potential chemotherapeutic agent in man may be deduced from the present study. Absorption is slow and excretion is so rapid that limitation of fluids may be essential to maintain an adequate blood level. However, the fact that the drug is not acetylated means freedom from kidney complications and freedom from the toxic manifestations associated with the use of diaminodiphenylsulphone.

The record of clinical trials concurrently appearing in the press should enable a preliminary assessment of the toxicity and therapeutic status of the drug to be made.

Summary

1. The chemical and physical properties of 4 : 4′-bis(γ-phenyl-*n*-propylamino) diphenylsulphone-

tetrasodium sulphonate, given the trade name of "sulphetrone," together with its absorptiometric estimation in body fluids, are described.

2. The acute toxicity of the drug has been investigated in mice and dogs and its chronic toxicity in rabbits.

3. A haemolytic anaemia, an anaemia of iron lack, and an anaemia of nutritional origin arising in the course of chronic sulphetrone administration in rabbits have been investigated and the conditions for their successful treatment indicated.

4. The specific goitrogenic effect, shared by all sulphonamide drugs, has been investigated and shown to be of slight degree.

5. In acute experiments sulphetrone given orally or parenterally raises the alkali reserve of the plasma in the rabbit and the dog. The probable mechanism is discussed. When the drug is administered over a period of time equilibria are established.

6. On the basis of these experiments, together with studies of the influence of the drug on normal growth of rats, it is concluded that sulphetrone is virtually non-toxic in acute experiments, and has low toxicity in prolonged ones.

7. Although exceedingly soluble in water, sulphetrone is slowly absorbed from the intestinal tract; most from the small intestine, little from the large.

8. The drug is not conjugated in the experimental animal or in man.

9. Sulphetrone penetrates all tissues, with the exception of brain, very rapidly, and is present in them to about the same concentration as in blood. It passes into the cerebrospinal fluid much more slowly than the simple sulphonamides do.

10. Sulphetrone has no action on smooth muscle, heart, blood pressure, or respiration.

11. Antibacterial *in vitro* studies, in the presence of blood, show sulphetrone to approach the efficiency of its parent substance diaminodiphenylsulphone against two strains of *M. avium*, two strains of *M. tuberculosis* var bovis, and three strains of *M. tuberculosis* var hominis.

12. The drug is rather more effective against β-haemolytic streptococcus infection in mice than sulphanilamide, but, unlike the parent substance, diaminodiphenylsulphone, it is ineffective against a pneumococcus infection.

13. In an *in vivo-in vitro* test, blood from a guinea-pig previously given parenteral sulphetrone inhibits, *in vitro*, strains of virulent mycobacteria.

14. The pharmacological properties of sulphetrone suggest that it may prove effective in the treatment of experimental tuberculosis in the laboratory animal, and that its administration to man, in large doses for protracted periods, is a practical possibility.

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THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS WITH SULPHETRONE*

BY

GEORGE BROWNLEE AND C. R. KENNEDY

From the Wellcome Physiological Research Laboratories, Beckenham, Kent

(Received June 7, 1947)

"Sulphetrone" is the registered name for bis(γ-phenyl-*n*-propylamino) - di - phenylsulphone-tetrasodium sulphonate, the pharmacology and chemotherapy of which have been recently reported (Brownlee, Green, and Woodbine, 1948). Sulphetrone has a low toxicity and an antituberculous efficiency approaching that of its parent compound, diaminodiphenylsulphone; it is also curative in infections due to β-haemolytic streptococci. Moreover it satisfies the requirements of an *in vivo–in vitro* comparison with diaminodiphenylsulphone; the concentrations of these substances in guinea-pig blood after massive intraperitoneal injections are capable of inhibiting the growth of virulent strains of *Mycobacterium tuberculosis*. The present report is concerned with the treatment of experimental tuberculosis of both human and bovine types in the guinea-pig.

Methods and Materials

Animal management

In carrying out screening tests of chemotherapeutic agents against *M. tuberculosis* there are two essentials: protection of staff from the risks of infection, and prevention of intercurrent infection in the animals under experiment; these can be achieved only by continuous and vigilant cleanliness on the part of a co-operative and dependable staff of helpers. It is an advantage to adopt a rigid daily routine which is on record available to and approved by all concerned. The animal room is 8 ft. by 30 ft., with transom windows along the long side and with a single entrance set at the opposite end of the long side. In order to assist ventilation it consists of a frame filled with fine copper gauze, with which the windows are also screened. The door opens into a vestibule "lock" enclosed by frame-work and door of wire gauze. This arrangement reduces the potential passage of all possible vectors and serves in addition to emphasize the "isolation" nature of the work. Walls are of breeze-block and plywood, filled, and glossy enamelled, and the ceiling is of "cellotex," and is similarly treated. Switches, cable, and light fittings are waterproof. The floor, of concrete, is treated with spindle-oil at two-monthly intervals, and is scrubbed weekly. The whole is washed down at two-monthly intervals. Temperature is held approximately at 20° C. and is controlled by hand-controlled steam-heated radiators and an extractor fan.

Guinea-pigs are housed six to a galvanized-wire cage fitting in a sheet-metal tray. The floor space per animal is 48 sq. in. The animals are identified by a combination code of natural colouring and artificially applied dye. White-enamelled metal hook-on labels have been found the most satisfactory after trials of many other types and of other devices. Food containers of tin-plate are non-spill and consist of an annular moat of 8-in. diameter with a central hollow projecting pillar to prevent soiling, and hold 250 g. of dry diet. Glazed non-spill water-pots holding 120 c.c. of water, are refilled three times daily. Absorbent litter is large wood shavings which scatter less than does sawdust. Weights are recorded weekly with a spring-pan balance weighing 1,000 ± 10 g. Changing to clean, previously autoclaved cages is done daily, except Sunday. Soiled cages, together with feeding-troughs and water-pots, are steamed before cleaning, and for this and similar purposes a double-door autoclave connects the animal room to the cage-cleaning room.

Guinea-pigs

In recent years guinea-pigs bred and reared on our own farm have been used. This has created a further problem since these animals, in addition to being much less prone to intercurrent infection, are more resistant to artificially induced tubercle infections than are the guinea-pigs of commerce.

* The development of sulphetrone is part of the programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain, Limited. The product was made and will be issued by the Wellcome Foundation, Ltd.

Diet

The dry, finely ground diet is based on that of Coward (1937) adapted to guinea-pig requirements. It is made up as follows:

Tubercle Diet No. 2

Yellow maize, whole, ground fine ...	700
Wheat, whole, ground fine	250
Milk, winter, dried (Glaxo)	50
Casein (B.D.H.)	90
Yeast, dried (Pharmaco-Chem. Prod.) ...	150
Salt mixture	12

The salt mixture has the following composition: manganese sulphate 0.1, potassium iodide 1.0, ferric citrate 1.0, sodium chloride 60.0, and calcium carbonate 1,000.0. The ingredients are mixed mechanically, and drug, when added, is mixed separately. The rate of consumption of this diet is 30–40 g. daily in guinea-pigs weighing 450–500 g., and their diet is supplemented with 100 g. of greenstuff.

Mycobacterium tuberculosis strains

The human strain CN. 271 (H. 37) is the virulent Saranac strain obtained from the Trudeau Sanatorium, N.Y., in 1931 and maintained on synthetic fluid medium by implant transfer at 21 days (Steenken and Gardner, 1946). In our hands this strain is moderately virulent. The bovine strain CN. 858 (A.N. 5 Weybridge) has proved to be apparently stable, by virulence titration, since its acquisition in 1943. It is highly virulent. In recent years both strains have been kept by Mr. H. Proom in a dried form which may prove to be the method of choice for maintaining virulence.

Grown on a solid egg-medium (Petragnani) for 14 days, suspensions are prepared by mechanically shaking with distilled water in a stainless steel bottle with steel balls and adjusted to 0.5 mg. per c.c. by reference to a previously standardized vaccine.

The estimation of sulphetrone in body fluids

The estimation of sulphetrone in body fluids has been described (Brownlee *et al.*, 1948) and is based upon diazotization and coupling to N(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939). The essential differences lie in the dilution of 1 in 15 and the concentration of acid, both of which are critical. Blood is obtained from the ear of the guinea-pig, from which, with patience and practice, up to 0.5 c.c. may be drawn into a clean heparinized capillary tube.

In Fig. 1 are given the blood-concentration time curves obtained with 1, 2, and 4 per cent of sulphetrone in the powder diet, and also the curve corresponding to a 2 per cent diet when greenstuff is fed in addition.

FIRST EXPERIMENT. *Bovine Strain CN. 858*

Forty young adult guinea-pigs of mixed sexes, weighing 450–500 g., were inoculated deeply into the right thigh muscles with 0.25 mg. of a fourteen-day-old sub-culture of bovine tubercle bacillus, CN. 858. The course of the infection was rapid, so that by the end of the second week glands were much enlarged and several animals had open abscesses. On the fifteenth day the animals were divided into two groups each of 20 animals, one to serve as control and the other to be the drug-treated group. Thereafter each animal in the latter group received in its food an estimated dose of 600 mg. of sulphetrone for the duration of the experiment. The blood sulphetrone concentration corresponding to this daily dose gave a mean value of 5 mg.±4 S.E. for weekly estimates during the course of the experiment.

RESULTS

Survival time.—A record of the weights and survival times of the animals is given in Fig. 2, from which it will be seen that the first animal in the untreated group died at 35 days and the last at 77 days after infection. About three-quarters of the animals were dead by 50 days. At a time when 50 per cent of animals in the control group were dead, 25 per cent were dead in the treated group; when all controls were dead, 25 per cent of the treated animals were still alive. The last animal in the drug treated group died 237 days after infection and 160 days after the last of the untreated animals had died. The average survival time after infection was 45 days in the untreated group and 77 in the treated group.

Necropsy studies.—Post mortem examination showed gross tuberculosis in 19 out of 20 animals in the untreated group, and one animal with severe tuberculosis. The nature of the infiltration was confirmed by histological examination and the organism was recovered by culture on solid egg-medium (Petragnani). In the drug-treated group of 20 animals, 9 showed gross, 5 severe, and 5 moderate tuberculosis; in one the only sign was a minute abscess at the site of injection from which the organism was recovered. The nature of the disease was confirmed by histological examination and the organism was recovered in 19 out of 20 animals.

The extent of the disease in the animals of both groups is shown pictorially in Fig. 3.

SECOND EXPERIMENT. *Human Strain CN. 271 (H. 37)*

Forty-five young adult guinea-pigs of mixed sex, weighing 450–500 g. were inoculated intramuscularly deep into the right thigh with 0.25 mg. of a 21-day-old culture of CN. 271 (H. 37). Fourteen days after infection the animals were divided

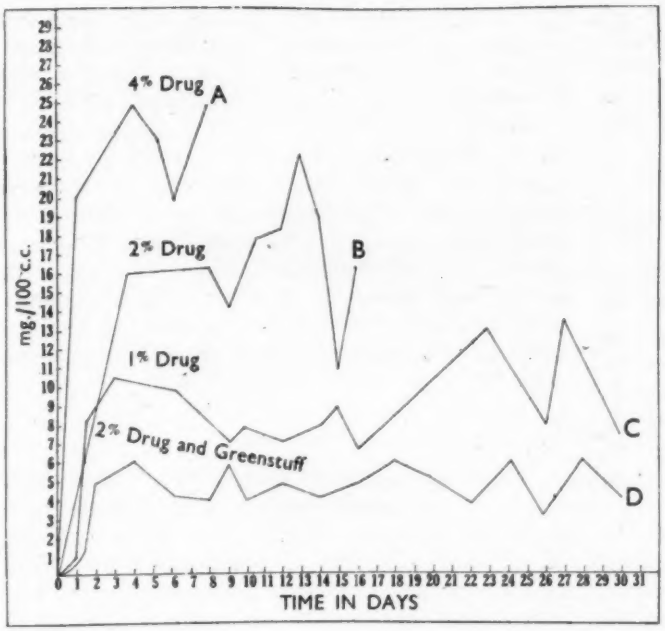

FIG. 1.—Blood concentration-time curves after administration of sulphetrone in the diet. Each curve is the average of estimates on a group of five guinea-pigs weighing 450–500 g. A, sulphetrone 4 per cent, equivalent to 1.4 g. daily; B, 2 per cent, or 0.7 g. daily; and C, 1 per cent, or 0.35 g. daily. In addition to 2 per cent drug in diet group D received 100 g. of greenstuff daily.

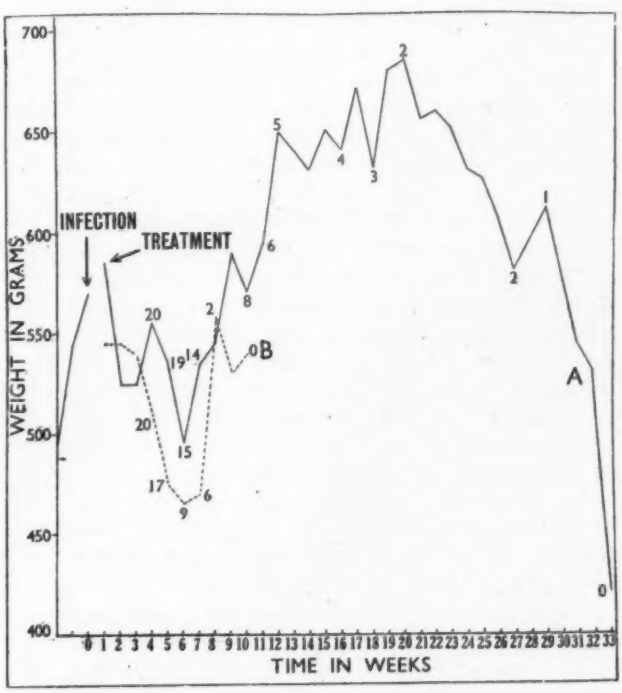

FIG. 2.—Weights and survival times of guinea-pigs after intramuscular infection with a bovine strain of tubercle bacillus. A, treated with sulphetrone; B, untreated. The figures on the graphs refer to survivors.

into two groups, one of 21 animals as a control, and the second of 24 animals as the drug-treated group; the latter group received 2 per cent of sulphetrone in the diet. The blood sulphetrone concentration corresponding to this daily dose, derived from weekly estimates made during the course of the experiment, was 4.8 mg. ± 2.8 S.E.

Results

Survival time.—There was a considerable difference in the survival times of the untreated and treated groups (Fig. 4). When the last of the controls had died at 154 days only 25 per cent of the group that had received sulphetrone had died. In only one guinea-pig of the untreated group was the cause of death possibly not due to tuberculosis. At the 154th day the 18 survivors in the drug-treated group were divided into two groups, one called drug-continued group, which continued to receive drug until the experiment terminated at 61 weeks when the last survivor was killed, and the other a drug-discontinued group from which drug was withdrawn. These two groups afford an interesting parallel. Within the drug-discontinued group deaths occurred regularly until the last animal died on the 280th day, or 126 days after the last animal in the control group had died. At this stage 50 per cent (4/9) of the animals in the drug-continued group were still alive.

Necropsy studies.—The extent of the disease in both groups of animals is shown pictorially in Fig. 5. Post-mortem examination showed gross tuberculosis in 13 out of 21 animals in the control group, five animals had severe tuberculosis, two moderate, and one in which the infection was minimal. The gall bladder and the small intestines of this animal showed the appearance characteristic of salmonella infection and there seems little doubt that this was the cause of death. The recovered organism was identified by Mr. Proom as *Salmonella bovis-morbificans.* The extent of the tuberculosis in all the drug-treated animals was very much less than in the untreated group. In the same period of 154 days in which all the controls died, three drug-treated guinea-pigs died with gross tuberculosis, one with severe and two with moderate lesions. Within the drug-discontinued group of nine animals, three showed gross tuberculosis, three severe, and three moderate. Within the drug-continued group two had gross, and four moderate tuberculosis, and in one animal the disease was minimal. The organism was recovered in all cases by culture methods.

Histology.—Histological studies confirmed the extent and nature of the disease. Not only was its distribution and extent in the treated group very much less but the numbers of acid-fast organisms were noticeably less. In addition there was much evidence of healing in lung, liver, spleen, and glands. The numerous regressive lesions with fibroblastic changes, many of them calcified, were impressive (Figs. 6 and 7). This change was observed also in lymph nodes in a number of animals. There was more tuberculosis of a progressive nature in the drug-discontinued group than in the drug-continued group, but here again there was impressive microscopic evidence of healing. The picture was that of a resurgence of tuberculosis which had been repressed by the drug; in the lungs this had the appearance of miliary tuberculosis (Fig. 7).

Discussion

The design of an animal experiment calculated to assess the therapeutic worth of a potential anti-tuberculous agent for man is a critical exercise fraught with difficulty. Tuberculosis in the experimental animal is a very different disease from tuberculosis in man in whom it is frequently characterized by an insidious onset, a chronic course, and a liability to relapse. Yet, the ultimate goal may be simply stated as the elimination of virulent tubercle bacilli, a point on which there is universal agreement (Feldman, Hinshaw, and Mann, 1944). The absence, to date, of a substance with this ideal requirement has dictated the evolution of other standards of comparison. These have been the comparison between survival-times of drug-treated and untreated groups of animals, a comparison of the nature and extent of tuberculous lesions in the organs of predilection, and a comparison between pieces of liver obtained by biopsy and at the final post-mortem examination. Ideally, a standard of comparison such as diaminodiphenylsulphone is included in the test. In a series of classical reports, Feldman and his collaborators (1940, 1942, 1943, 1944, 1945) have evaluated the status of promin and diasone, derivatives of diaminodiphenylsulphone, and promizole. Probably by reason of the exploratory nature of their first experiments these workers adopted the use of what may be called a minimal lethal infection, using the virulent Saranac strain of H. 37, and examined the treated animals at the time when the last control animal had died. This kind of experiment presents an effective anti-tuberculous agent in its most favourable light. Nevertheless, this is an entirely satisfactory practice when comparisons are made, especially when taken together with the biopsy control technique, and it has enabled Feldman and

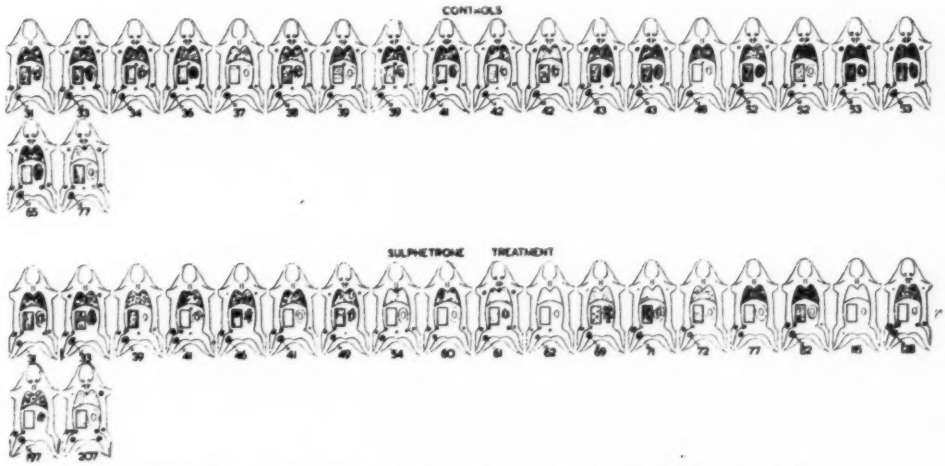

FIG. 3.—*First experiment.* Guinea-pigs infected with 0.25 mg. of the virulent bovine strain of tubercle bacilli CN. 858. A pictorial record of the gross amount and distribution of tuberculosis seen at death in untreated and sulphetrone-treated animals. The number beneath an animal shows the days of life after infection. Although observed until death from tuberculosis, there is less gross disease in the treated group.

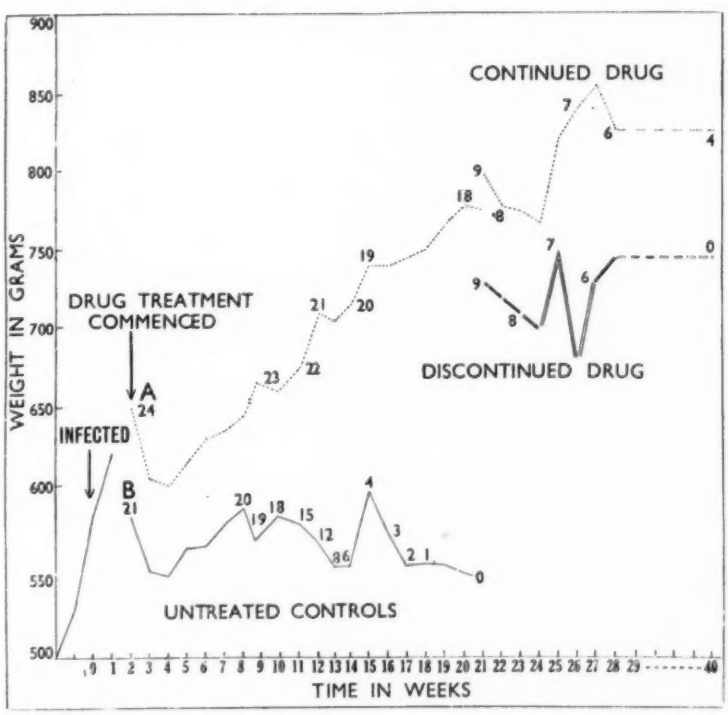

FIG. 4.—*Second experiment.* Weights and survival times of guinea-pigs after intramuscular infection with a human strain of tubercle bacillus. A, treated with sulphetrone; B, untreated. When all controls had died, the 18 survivors in group A were subdivided to give a drug-continued group and a drug-discontinued group. The figures on the graphs refer to survivors.

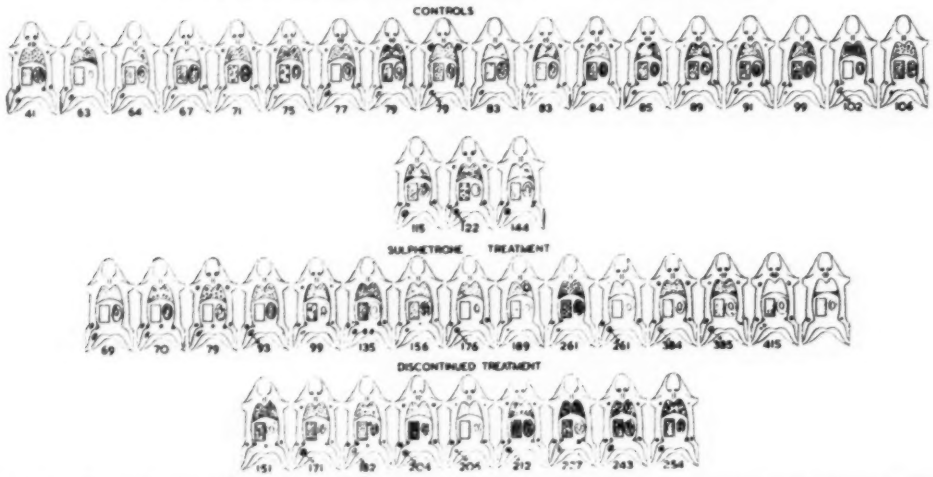

FIG. 5.—*Second experiment.* Guinea-pigs infected with 0.25 mg. of the virulent human strain of tubercle bacilli CN. 27i (H. 37). A pictorial record of the gross amount and distribution of tuberculosis seen at death in untreated and treated animals. The number beneath an animal shows the days of life after infection. Although observed until death from tuberculosis, there is less gross disease in the treated group.

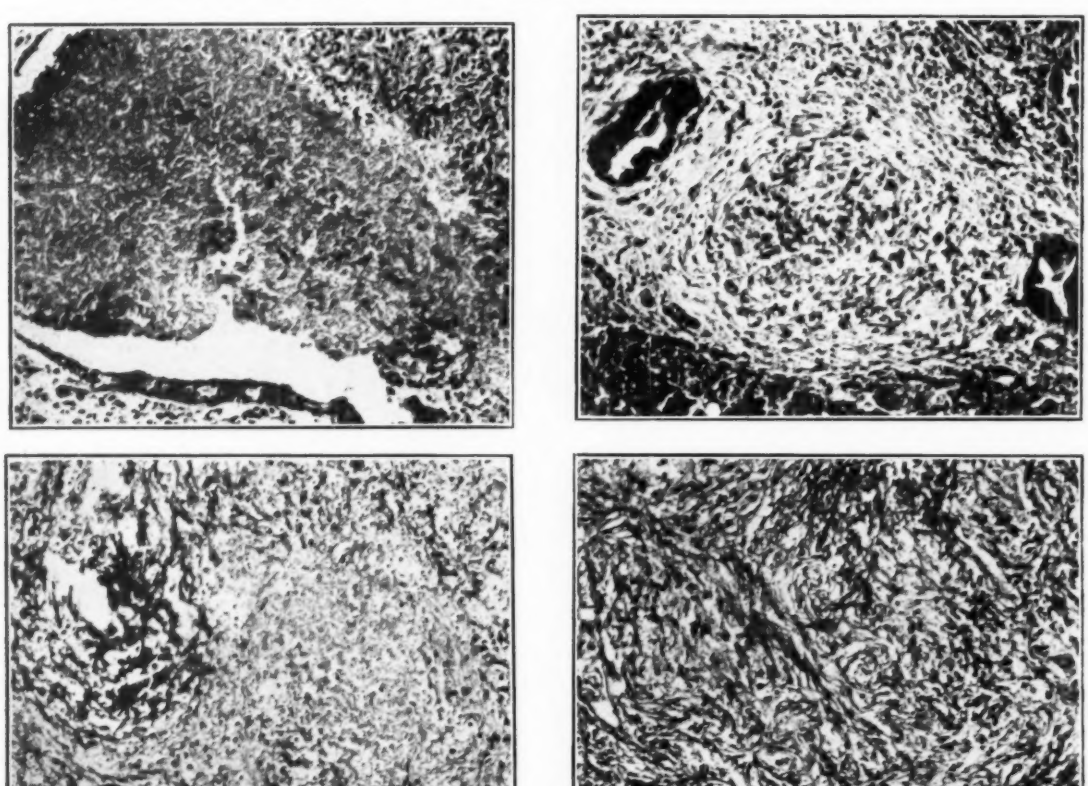

FIG. 6.—*Second experiment, H.* 37, *human strain.* (Top left) Area of progressive destructive tuberculosis in the liver of an untreated guinea-pig which died 143 days after infection with tubercle bacilli (× 66). (Top right) Fibrotic healing tubercles in the liver of a guinea-pig treated for 384 days with sulphetrone (× 66). (Bottom left) Progressive destructive tuberculous area in an inguinal lymph node of an untreated infected guinea-pig which died 143 days after infection (× 123). (Bottom right) Non-progressive, markedly fibrotic involution in an inguinal lymph-node of a guinea-pig treated for 384 days with sulphetrone.

his collaborators to evaluate the status of chemotherapeutic antituberculous drugs. It is their opinion, with which we agree, that the sulphone drugs are not capable of eliminating virulent organisms from the organs of predilection in the experimental animal.

In making this first report of the antituberculous activity of sulphetrone it seemed proper to record tests which presented the drug in its least favourable light

In the experiments reported here, the amounts of the virulent strains inoculated were large, and in addition to an experiment with a human strain, a test with a bovine strain is reported. Also, the animals were observed until death. In another place comparative tests with other drugs will be reported.

Sulphetrone is a derivative of diaminodiphenylsulphone of low, acute and chronic toxicity. As will be reported elsewhere, its lack of toxicity is such that doses equivalent to those given to guinea-pigs and sufficient to give blood concentrations of 5 to 7.5 mg. per 100 c.c. have been administered to man for continuous periods of 12 or more months. In this respect it differs from promin and diasone whose toxicity is such that they may be given for only short and intermittent periods. Since others have considered that the therapeutic action of promin and diasone may be due to some extent to their degradation to diaminodiphenylsulphone, it is interesting that with sulphetrone the evidence, both chemical and pharmacological, is that this breakdown does not occur.

In the first test the experimental conditions, both in respect to the amount of the virulent infecting organism and to its bovine origin, were severe. Nevertheless, the group of infected guinea-pigs receiving 2 per cent of sulphetrone in their diet showed a significant prolongation of survival-time. For example, at a time when half of the control animals were dead, only one-quarter of the treated group were dead ; when all control animals were dead, one-quarter of the treated group were still alive. Expressed in terms of average survival days the figure for the treated group was 77 days, and for the untreated group 45 days.

The inoculum used in the second experiment was the large injection of 0.25 mg. of the human virulent Saranac strain H. 37. In this instance the protection given by drug treatment was large. For example, when the last of the control animals had died, three-quarters of the treated animals were still alive, and as Fig. 4 shows, were in very good physical condition. In contrast, the animals from the control group showed gross tuberculosis, and histological examination revealed the presence of

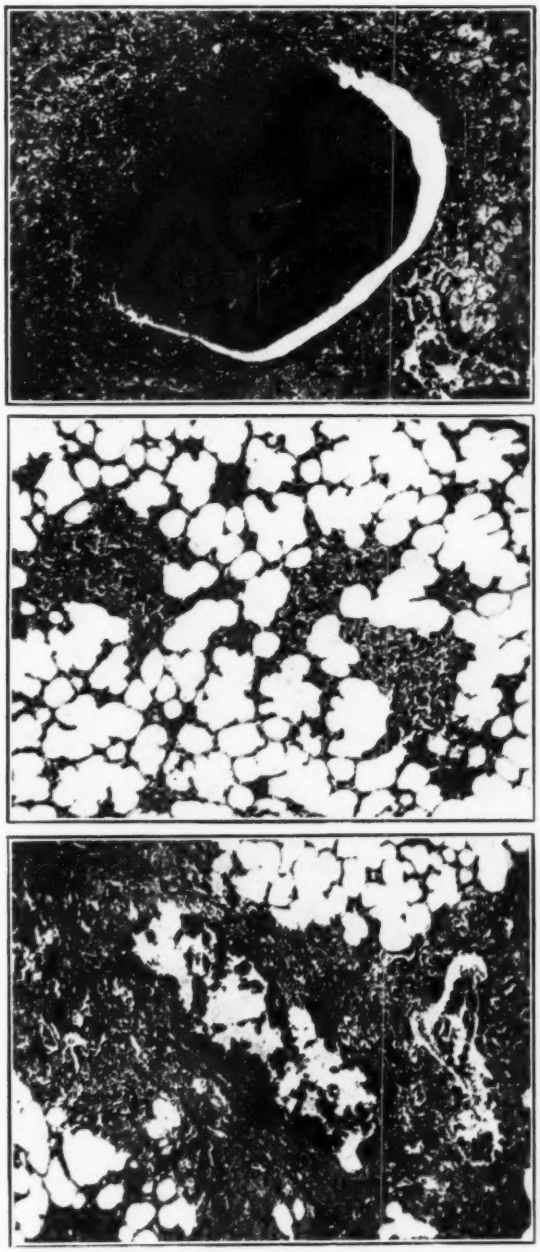

FIG. 7.—*Second experiment, H. 37, human strain.* (Top) Severe necrotizing progressive tuberculosis in the lungs of an untreated guinea-pig which died on the 143rd day after infection with tubercle bacilli (×123). (Centre) Discrete tuberculous nodules consisting of coalescent young miliary tubercles from the lungs of a guinea-pig treated for 154 days with sulphetrone and then treatment discontinued. Died on the 204th day (×123). (Bottom) Calcified fibrotic nodule in the lungs of a guinea-pig treated with sulphetrone until death on the 189th day (×123).

numerous tubercle bacilli. By dividing the survivors into two groups, one in which drug was continued and one in which drug was withdrawn, a very significant observation was made. The order of protection conferred by the drug was continued in one group and withheld in the other. Expressed in figures, it is shown that at the time when all members of the drug-discontinued group were dead, one-half of the drug-continued group were still alive. Throughout the entire drug-treated group macroscopic evidence obtained at necropsy showed very much less tuberculosis than in the untreated group. This was confirmed by histological examination and amplified by the observation that acid-fast organisms were very much less in number than in the untreated group, where they were numerous. The most significant histological evidence was the repeated finding of healed tuberculous lesions in spleen, liver, lungs, and lymph nodes ; often they were calcified, particularly in lungs and lymph nodes.

In both experiments there is evidence of a therapeutic effect present only in the groups treated with sulphetrone. With both the bovine and the human strains the results suggest that sulphetrone exerts a retarding effect on the progressive nature of established experimental tuberculosis in the guinea-pig. It should be remembered that the conditions of the tests gave the infection the optimum advantage. In addition, in the experiment using the human strain, there was impressive histological evidence of a reversal from progressively destructive disease to one in which the morbid process was retarded ; there was also evidence of resolution and calcification.

The therapeutic effect is shown most markedly by the difference between percentage survivors in the treated and untreated groups, but it is evident that sulphetrone, in common with other similar chemotherapeutic agents, is incapable of eliminating the causative organism.

Summary

1. The chemotherapeutic value of 4,4′-bis(γ-phenyl - *n* - propylamino) - diphenylsulphone tetrasodium sulphonate, given the trade name of "sulphetrone," in treating experimental tuberculosis in guinea-pigs is described.

2. When sulphetrone was added to the diet in 2 per cent, average blood concentrations of 5 mg. per 100 c.c. were found throughout the experiments. The average amount of drug eaten was 0.6 g. daily.

3. In an experiment in which two groups of 20 animals were infected with a heavy inoculum of the virulent bovine strain of tubercle bacilli CN. 858, the survival time of the drug-treated group was prolonged. The average survival time was 45 days in the untreated group and 77 days in the treated group.

4. In a second experiment in which the infection was a heavy inoculum of the human virulent H. 37 strain (CN. 271), the treated group of 24 animals survived considerably longer than the untreated group of 21 animals.

5. There was a resurgence of tuberculosis of a miliary kind in a group of guinea-pigs in which drug treatment was stopped.

6. In the drug-treated animals which died there was impressive histological evidence of healed tubercles.

7. Under the severe conditions of the experiments, sulphetrone was capable of exerting a suppressive effect on the progressive nature of the experimental infections. Sulphetrone is bacteriostatic and, in common with other derivatives of diaminodiphenylsulphone, is not capable of eliminating the infective organism from animal tissue.

We are indebted to Dr. David Trevan for the histological preparations and for assistance with the pathology. He is also responsible for the photomicrographs.

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Brit. J. Pharmacol. (1948), **3**, 37.

THE CHEMOTHERAPEUTIC ACTION OF STREPTOMYCIN, SULPHETRONE, AND PROMIN IN EXPERIMENTAL TUBERCULOSIS*

BY

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(Received July 18, 1947)

The accumulated evidence from both experimental chemotherapeutic and clinical sources makes it possible to say that while streptomycin approaches closer than any other known chemotherapeutic agent to the concept of a completely effective antituberculous drug, it fails to eliminate unconditionally the causative organism. Of significance in this connection is the clinical evidence presented elsewhere (Madigan, Swift, and Brownlee, 1947b) that the antibiotic appears to be most effective in rapid progressive disease, and least effective in chronic disease. The metabolism of the organism seems to be implicated in these effects, since in young dividing cells streptomycin is markedly effective, while it is ineffective against old resting cells. It appears to be significant that when smaller infections of tubercle bacilli are used (0.1 mg. H.37; Feldman, Hinshaw, and Mann, 1945) the results are better than when larger infections are used (1.0 mg. A.27; Smith and McClosky, 1945).

From theoretical considerations of what is known of the mode of action of diaminodiphenylsulphone drugs, it is not surprising that sulphones and streptomycin show a synergism of action. The synergism between streptomycin and promin demonstrated by Smith and McClosky (1945), and between streptomycin and diasone by Callomon, Kolmer, Rule, and Paul (1946), is of a high order and, in view of the relative failure of streptomycin by itself, suggests the possibility of combined clinical therapy. Sulphetrone, the pharmacology and chemotherapy of which has been recently described (Brownlee, Green, and Woodbine, 1948), is a diaminodiphenylsulphone derivative of low chronic toxicity which is effective in experimental tuberculosis and which may be used to maintain effective blood concentrations in man for continuous periods of 12 or more months (Brownlee and Kennedy, 1948).

The present report describes a comparison between the chemotherapeutic antituberculous activity of streptomycin, sulphetrone, promin, and combined streptomycin and sulphetrone.

Methods and Materials

A recent account (Brownlee and Kennedy, 1948) has appeared of the methods adopted in the management of guinea-pig tests. The same report should be consulted for the composition of the dry powdered Tubercle Diet No. 2 which constitutes the basic ration.

Mycobacterium tuberculosis strains

The strain CN.844 used in the present tests is a virulent human strain isolated in 1942 from a case of tuberculous adenitis, and maintained on synthetic fluid medium by implant transfer at 21 days. This virulent strain gives rise to a more chronic type of disease than the virulent Saranac strain CN.271 (H.37) previously used. This change was dictated by a temporary loss of virulence in the H.37 strain, a feature discussed by Steenken and Gardner (1946), who make suggestions for avoiding this complication.

Streptomycin

The sample of streptomycin sulphate used in this test was drawn from W.F. Batch 17 and is of 33 per cent purity. The chemotherapy and pharmacology of this batch together with the method of assay have been described (Madigan *et al.*, 1947a). The potency of streptomycin is stated in terms of weight of pure streptomycin base $C_{21}H_{37}N_7O_{12}$ (mol.wt.579) and is derived by assay with a working standard of streptomycin sulphate ($1\frac{1}{2}$ H_2SO_4; mol.wt.726) of known potency; it follows that streptomycin sulphate contains 79.8 per cent of base.

Sulphetrone

"Sulphetrone" is the registered name of the Wellcome Foundation Ltd. for 4:4′-bis(γ-phenyl-*n*-propylamino)diphenylsulphone tetrasodium sulphonate. It

* The development of sulphetrone is part of a programme of work on antituberculous compounds carried out by the Therapeutic Research Corporation of Great Britain.

is available as a cream-coloured amorphous powder containing 9–10 per cent of water. Assays in this report are in terms of anhydrous material. Promin, 4:4′-diaminodiphenylsulphone-N, N′-didextrose sodium sulphonate, is a cream-coloured product containing, when freshly prepared, 10 per cent of water; assays are also given in terms of anhydrous material.

The estimation of drugs in body fluids

The estimation of sulphetrone in body fluids has been described (Brownlee, Green, and Woodbine, 1948), and is based upon diazotization and coupling to N(1-naphthyl)-ethylenediamine hydrochloride (Bratton and Marshall, 1939). The essential points are an over-all dilution of 1 in 15 and the concentration of acid, both of which are critical. Promin was estimated by the same method, which gave recoveries of some 75 per cent, to which a correction factor was applied. Streptomycin in blood was estimated by the slide technique of Fleming (1943), using a susceptible haemolytic strain of *Escherichia coli* CN.1360. Blood, in quantities up to 0.5 c.c., was obtained from the ear of the guinea-pig by drawing it into a clean heparinized capillary tube.

Experimental

Eighty-three young adult guinea-pigs of mixed sexes, weighing 650–700 g., were inoculated deeply into the right thigh muscles with 0.25 mg. of a fourteen-day-old sub-culture of human tubercle bacillus, CN.844. Seventeen days after infection the animals were tested for their reaction to an intradermal injection of 0.1 c.c. of 1/10,000 Old Tuberculin. All animals gave a positive reaction; eight reactions were classed as mild. Tuberculin tests were repeated at the 15th and 23rd week of infection. On the twenty-first day, liver samples were obtained from two animals from each group by biopsy under pentobarbital sodium anaesthesia. On the following day the animals were divided into five groups, four of 18 animals and a control group of 11 animals.

Streptomycin treatment

One group of 18 guinea-pigs received the equivalent of 10 mg. of pure streptomycin base daily. Four four-hourly intraperitoneal injections each of 2.5 mg. of streptomycin base contained in 0.25 c.c. of pyrogen-free distilled water were given with aseptic precautions at 8 a.m., 12 noon, 4 p.m., and 8 p.m.; no drug was given during the intermediate 12 hours. Treatment was continued for 168 days, during which time each guinea-pig received 1.68 g. of the antibiotic. Concentrations of streptomycin per c.c. of plasma were estimated at intervals throughout the test and the average figures 1 hour after injection were 7.0 μg. $\pm$1.0 (S.E. of mean of 60 observations), and at 3.5 hours 4.0 μg. $\pm$1.0 (72); at 4 hours no streptomycin could be detected in the plasma. Two animals in this group died from intraperitoneal haemorrhage as a result of accidental puncture of the spleen, one at the 7th week after infection, and one at the 8th week.

Combined streptomycin and sulphetrone treatment

In addition to treatment with 10 mg. of streptomycin base daily, a group of 18 guinea-pigs also received 2 per cent of sulphetrone incorporated in the dry diet.

Plasma concentrations of streptomycin were estimated at intervals throughout the test, and the average figures were similar to those of the streptomycin-treated group; these were 7.0 μg. $\pm$1.5 (90) at 1 hour, and 4.0 μg. $\pm$1.0 (90) at 3.5 hours. The average blood sulphetrone concentration per 100 c.c. was 5.1 mg. $\pm$ 3.8 (90).

Sulphetrone treatment

A group of 18 guinea-pigs received 2 per cent of sulphetrone in the dry diet. This corresponded to an average drug intake of 600 mg. daily and resulted in an average blood sulphetrone concentration per 100 c.c. of 5.9 mg. $\pm$3.5 (86).

Promin treatment

A group of 18 animals was treated with promin incorporated to a concentration of 0.5 per cent in the diet. The average drug intake was 150 mg. daily and gave a blood promin concentration per 100 c.c. of 3.5 mg. $\pm$2.2 (60).

Control group

Eleven animals constituted the control group and received no treatment.

Results

Survival times

Inoculation with 0.25 mg. of the CN.844 strain established a slow chronic type of infection well suited to the comparison; for this reason only three animals in the control group of 11 died during the experiment of 168 days, and these from gross generalized tuberculosis. In the same period of time two animals died in the streptomycin treated group at 46 and 56 days owing to intraperitoneal haemorrhage as a result of needle injury to the spleen. Post-mortem examination revealed minimal tuberculosis. An unfortunate accident resulted in the death of five additional guinea-pigs of this group, on the 66th day, thus reducing the effective number of streptomycin guinea-pigs to eleven. Post-mortem examination of these animals showed no detectable lesions. Two animals in the combined streptomycin and sulphetrone group died with intraperitoneal haemorrhage owing to spleen injury; in both animals the tuberculous lesions were minimal. One animal found dead on the 168th day had no tuberculosis other than a small abscess at the site of injection and an enlarged inferior inguinal gland; the cause of death was believed to have been a concurrent salmonella infection. Four animals in the sulphetrone group

died ; in none of them was the tuberculous process so well advanced as to justify the view that death was due to tuberculosis. Within the promin group there were seven deaths: one of these, at 25 days, was due to a streptococcal pneumonia, minimal tuberculous lesions being present ; in the other six animals the tuberculosis was extensive enough to justify the belief that it had been the major cause of death, a contributory feature being the toxic nature of the drug ; guinea-pigs in this group were subdued and in poor condition.

Effects on weight

The influence of the drug treatment on the average weights of the guinea-pigs is shown in Fig. 1, where the effect of the four test substances and combination of substances is compared with the control group. Both groups receiving streptomycin by intraperitoneal injection show a loss of weight which lasts for some six weeks and is then followed by recovery. The fact that the final weight records show the greatest gains within this group suggests that the temporary loss of weight may have been caused by the repeated intraperitoneal injections at the commencement rather than by a specific toxic effect.

The group receiving sulphetrone gained in weight, and the group receiving combined sulphetrone and streptomycin, though they barely maintained their weight, were in good condition and more alert than any other group. The promin group lost as much weight as did the controls, with which they compared unfavourably in general condition. When the experiment ended their condition was exceedingly poor and suggestive of toxaemia.

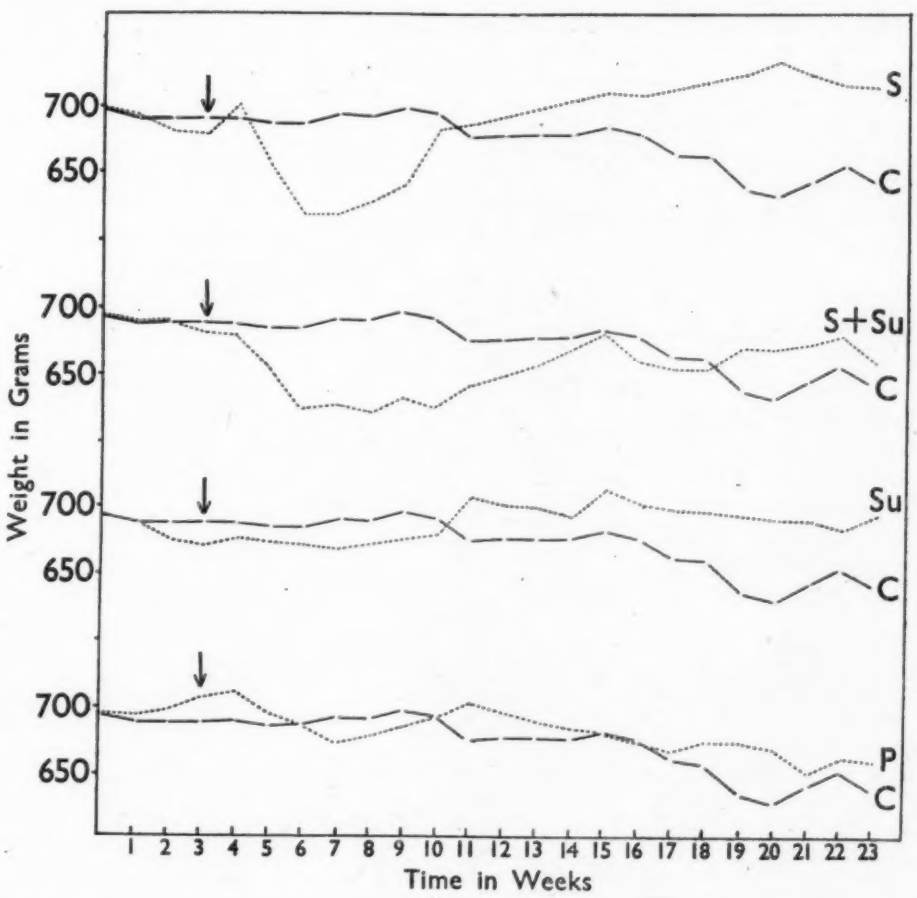

FIG. 1.—The influence of drugs on the average weights of groups of guinea-pigs infected with 0.25 mg. of a virulent strain of human tubercle bacilli. Drug therapy begun at arrow. S, streptomycin. S+Su, streptomycin with sulphetrone. Su, sulphetrone. P, promin. C, control untreated group.

Clinical

Differences between the treated and untreated groups, and within the treated groups themselves, were apparent by 90 days after infection. In the untreated group the superior and inferior inguinal glands were grossly enlarged. In the groups treated with streptomycin alone and with streptomycin and sulphetrone these glands were not enlarged, or only slightly so. This difference was less obvious between the sulphetrone and control groups and not evident at all in the comparison of the promin and control groups. The normal appearance of the groups on streptomycin alone and on combined therapy contrasted strikingly with the toxic appearance of the group treated with promin.

Tuberculin tests

Throughout the course of the experiment three tuberculin tests were made. The first, for the purpose of rejecting non-reactors, was on the 17th day; a second was made on the 105th day, and a third on the 147th day. On each occasion 0.1 c.c. of a 1 in 10,000 dilution of Old Tuberculin was injected intradermally and the subsequent reaction read at 72 hours. At the second test the severity of the reactions within the different groups appeared to be related to the extent of the disease suggested by the records of enlarged glands, losses in weight, and general health of the animals.

Reducing the clinical observations to a simple numerical basis gave the figures shown in Table I for both 105 and 147 days. The numerical notation is as follows: 1, negative; 2, query negative, or a needle injury; 3, weak positive such as mild diffuse staining of skin or a small reddened area of 3 mm. or less in diameter; 4, positive, without necrosis; 5, positive, with necrosis of mild limited appearance; 6, positive, with necrosis of moderate extensive appearance; 7, positive, severe reaction. The Table shows the mean numerical indices corresponding to degrees of severity of actions.

TABLE I

TUBERCULIN REACTIONS IN DRUG-TREATED AND UNTREATED GUINEA-PIGS

Group	Number of guinea-pigs	Mean numerical index of reactions ± S.E.	
		105 days	147 days
Streptomycin	12	3.8 ± 1.2	4.5 ± 1.6
Streptomycin + Sulphetrone ..	16	2.5 ± 0.9	3.7 ± 1.3
Sulphetrone ..	15	4.1 ± 1.4	4.5 ± 1.4
Promin ..	16	4.5 ± 1.3	4.9 ± 1.8
Control ..	11	5.2 ± 1.6	4.5 ± 1.4

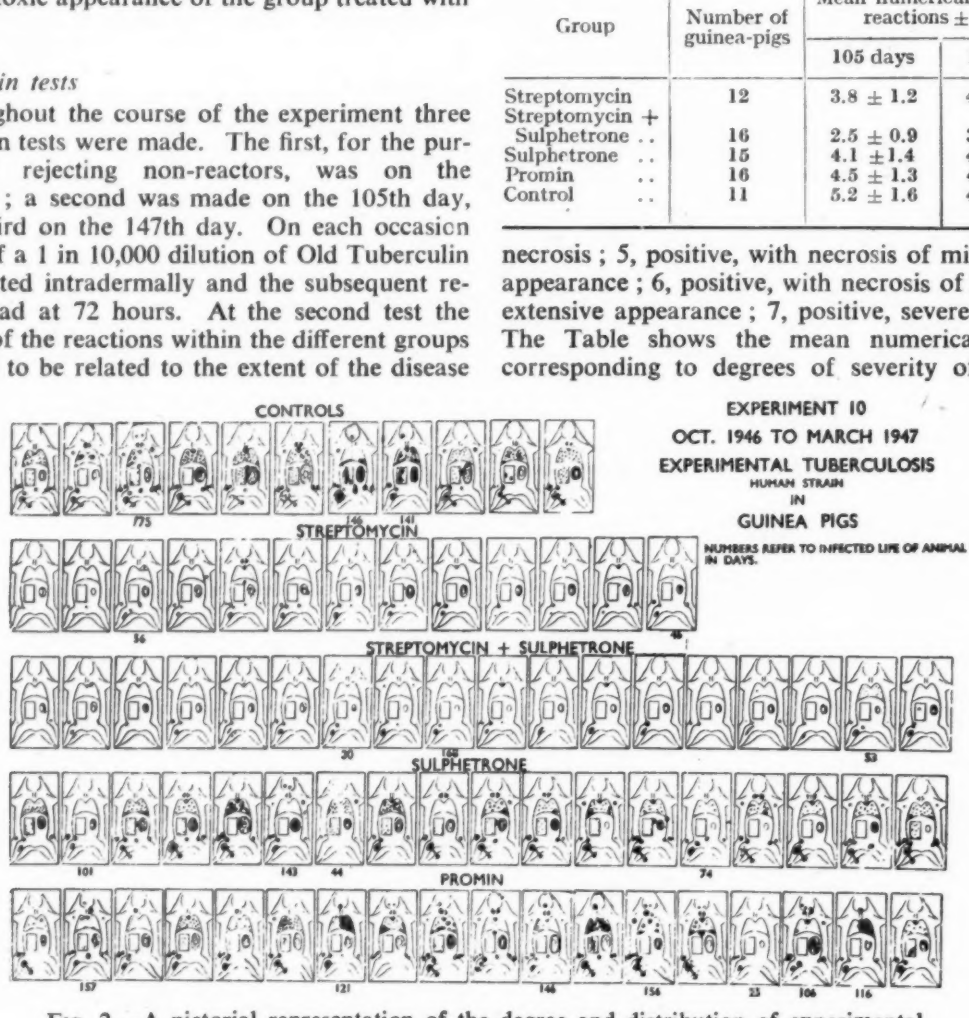

FIG. 2.—A pictorial representation of the degree and distribution of experimental tuberculosis in guinea-pigs *post mortem* 178 days after infection with 0.25 mg. of a virulent strain of tubercle bacilli. The drug-treated groups received 10 mg. streptomycin base daily, 10 mg. streptomycin base daily plus 2 per cent sulphetrone in the diet, 2 per cent sulphetrone in the diet, and 0.5 per cent promin in the diet. The control group was untreated.

Although the disease appears to have been progressive during the course of treatment, the comparative effectiveness of each drug in limiting the spread of the disease appears to have remained constant. Statistically, the differences are probably real.

The enlargement in the accessory glands also shows that the disease was progressive in all groups during the interval between the second and third tests. The decrease in the tuberculin reaction in the control group is probably explained by the common observation, both in animals and man, that in the course of progressive disease skin reactions often become less sensitive.

Necropsy studies

The experiment was terminated on the 178th day and the animals brought to post-mortem examination. We have become used to observing and recording differences between treated and untreated animals, but have not met previously such striking differences as were observed between the group which received combined treatment and the controls, and to a less extent between the streptomycin and control groups. In these treated groups both spleen and liver were normal in size and colour and tuberculosis was minimal in extent.

There was gross, extensive, and progressive generalized tuberculosis in all animals of the untreated group. The promin treated group showed, in general, markedly less tuberculosis, although in many animals spleens were grossly tuberculous. Within this group five animals had disease comparable to that seen in the control group, with extensive caseation and cavitation of the lungs. The sulphetrone group showed much less tuberculosis than the control group and less tuberculosis than the promin group. This was particularly evident in the spleens and liver.

The extent and distribution of the disease in the drug treated and the untreated groups is shown pictorially in Fig. 2 and numerically in Table II in which, following the excellent notation of Feldman (1943), scores are given for the extent and character of the tuberculosis observed macroscopically in spleen, liver, lungs, and glands.

TABLE II

AVERAGE SEVERITY AND DISTRIBUTION OF TUBERCULOSIS EXPRESSED NUMERICALLY

Group	Macroscopic examination of the tissues involved					
	Number of animals	Spleen (maximum 35)	Lungs (maximum 30)	Liver (maximum 25)	Site and lymph nodes (maximum 10)	Average index (maximum 100)
Controls	11	30.5	25.5	19	10	85.0
Streptomycin ..	13	13.9	4.6	0	9	27.5
Streptomycin and sulphetrone ..	18	9.4	2.5	0.5	8.3	20.7
Sulphetrone ..	18	20.0	18.6	9.4	9.4	57.4
Promin ..	18	24.2	22.2	11.7	10	68.1

Bacteriology

Post mortem the spleens were removed aseptically and divided for histological examination and examination for tubercle bacilli. For the latter purpose they were ground in a previously sterilized mortar in 6*N* HCl, spun, neutralized with a calculated amount of NaOH and seeded on to egg-medium. Acid-fast organisms of typical morphological appearance were recovered from all the control animals; within the promin group there were 16 out of 18 recoveries, with sulphetrone 12 out of 18, with streptomycin 4 out of 13, and from the combined therapy group 2 very scanty growths from 18 cultures. It was noticeable that the cultures obtained from the promin treated pigs grew luxuriantly, in contrast to all other treated groups.

Sensitivity of strain and cultures to drugs

The CN.844 strain when grown in Long's Synthetic Medium is inhibited by 0.5 μg./c.c. of streptomycin, by 65 μg./c.c. of sulphetrone, by 125 μg. of promin, and by a mixture of 30 μg./c.c. sulphetrone and 0.25 μg./c.c. streptomycin, in which mixture an additive, not a synergistic, effect is seen. Significant, in view of the therapeutic effects, was the finding that all strains recovered *post mortem* were unchanged in sensitivity to streptomycin and sulphone drugs.

Histology

Examination of histological material from the untreated control group revealed a uniform picture of severe, progressive infection. Confluent tuberculous interstitial pneumonia, with frequent caseation and many acid-fast bacilli, was seen in the lungs; the livers exhibited multiple tubercles spreading outwards from the portal tracts with many acid-fast bacilli in infarcted areas. Spleens had massive infarcts, haemorrhages, and coalescent small tubercles, mostly with caseous centres, and many acid-fast bacilli were present. The

lymph nodes were occupied by confluent caseating tubercles and generally acid-fast organisms were seen.

The treated groups differed, more or less, in the amount and distribution of the tuberculosis and in the numbers of acid-fast bacilli seen.

The promin group differed least from the controls. Involvement of the lungs was the rule, but there was evidence of localization and repair. Livers showed little frank tuberculosis, but there were extensive foci of recent necrosis ; acid-fast organisms were rarely seen. Spleens were extensively involved, with numerous tubercles, extensive infarction and congestion ; acid-fast organisms were rare.

The restraining powers of the sulphone drugs were carried a demonstrable step forward in the sulphetrone group. Lungs were less extensively involved, the lesions being limited at the most to patchy tuberculous interstitial pneumonia coalescing in areas into large tubercles, with rare necrosis ; at the least, there was congestion and oedema ; acid-fast bacilli were not seen. Livers showed small areas of miliary tubercles and an occasional fibrosing tubercle ; acid-fast bacilli were not seen. Spleens showed numerous small tubercles in a congested oedematous pulp but without acid-fast bacilli being identified.

The streptomycin group differed markedly from the controls. Only 5 out of 13 lungs showed tuberculous lesions : in two they were restricted to single fibrotic calcified tubercles. In only one animal was there a tuberculous lesion in the liver. With the exception of one animal in which the disease appeared to be non-progressive, tubercles were found in all spleens. Mostly the tubercles were fibrotic (5 cases), or calcified (7). Tubercles were seen in all the contiguous lymph nodes ; in three animals they were limited to small fibrotic areas

The group treated with streptomycin and sulphetrone differed remarkably from the controls and presented an impressive degree of protection even when compared with the streptomycin group. Only 6 out of 18 lungs showed tuberculous lesions ; in five of these the lesion was calcified and fibrotic. All lungs showed evidence of many healed lesions. In only one liver was a tubercle found and this was a small hardened epithelioid node. The spleens were involved in 14 out of 18 animals, but all the lesions were non-progressive, consisting of areas of diffuse fibrosis or of repressive lesions with calcification. Lymph nodes adjacent to the inoculation site were minimally involved in all animals ; all the lesions were non-progressive and consisted almost entirely of fibrous tissue with some calcification.

Discussion

Although incapable of eliminating the causative organism unconditionally, streptomycin is capable of modifying favourably the course of inoculation tuberculosis in the experimental animal (Smith and McClosky, 1945 ; Feldman, Hinshaw, and Mann, 1945 ; Youmans and McCarter, 1945). Viewed from the base-line laid down by experimental and clinical sulphone therapy the significant advance in efficiency made by this new antibiotic justified a careful clinical assessment. From the clinical reports now available it is apparent that the antibiotic is able to influence tuberculosis to only a limited extent and under certain conditions. Ironically enough these appear to be the conditions under which the bacillus is multiplying rapidly, for example in miliary tuberculosis. The importance of appreciating the reasons for these failures needs no stressing. Thus, Baggenstoss, Feldman, and Hinshaw (1947) record the death of 5 cases of miliary tuberculosis in spite of healing in the lungs, and they note that streptomycin could not be demonstrated in brain substance in which tuberculous lesions were found. Madigan, Swift, and Brownlee (1947b) had similiar failures with miliary tuberculosis to report, and in addition they could not record healing in phthisis of diverse origin. If the disease in the experimental animal is regarded as different from the diseases due to *M. tuberculosis* in man, this difference reflects the metabolic adaptability of the organism and it is to countering this aptitude that we must address our efforts. The acute experiments, here described, offer no evidence of adaptation of the organism to any of the chemotherapeutic agents ; significant in this connection is the record of Madigan *et al.* (1947b), that in chronic disease (phthisis) the organisms acquired resistance to streptomycin, while in acute infection (miliary tuberculosis) in which healing was seen the susceptibility of the organism was unchanged. Experimental evidence is presented by the same authors, that streptomycin is most effective against young dividing bacilli and ineffective against old resting cells. A similar observation is made by Middlebrook and Yegian (1946). A practical approach to the attack on the metabolic adaptability of the tubercle bacillus is that of Smith and McClosky (1945), who showed the synergistic action of streptomycin and promin in experimental tuberculosis ; Callomon, Kolmer, Rule, and Paul (1946) also demonstrated a similar effect with diasone and streptomycin.

The present report is concerned with the synergism of streptomycin and sulphetrone, a diaminodiphenylsulphone derivative which combines efficiency of action with freedom from the chronic toxicity associated with the use of promin and diasone. Groups of animals treated with promin, with sulphetrone, and with streptomycin enable a comparison to be made.

On the basis of survival time, change in weight, response to tuberculin tests, macroscopic evidence of gross tuberculosis *post mortem* or microscopic examination there is presented a uniform picture of the marked superiority of the combined therapy. The order of efficiency is streptomycin with sulphetrone, streptomycin, sulphetrone, and promin. It should be noted that oral promin exhibits the drug by its most toxic route and that the resulting blood levels are consequently low; this remains, however, a practical problem. It is interesting that a relation was found between the degree of protection and the severity of intradermal tuberculin reactions, an observation in contrast to that of Smith and McClosky (1945). A careful sifting of all evidence here presented leaves the impression that the disease was progressive in all groups, albeit at a much suppressed rate in those groups where protection was greatest. Nevertheless, with this implication in mind, the experimental effects produced by the combined streptomycin and sulphetrone therapy are believed to justify a careful clinical evaluation in selected cases. The need meanwhile is for improved antituberculous bacterial antibiotics, which will not induce resistant strains.

Summary

1. Four groups of 18 guinea-pigs, and one control group of 11, infected with 0.25 mg. of a human virulent strain CN.844 of *M. tuberculosis* were treated 22 days later with drugs for 168 days. One group received 0.5 per cent of promin in the diet, one 2 per cent sulphetrone in the diet, one 10 mg. of streptomycin parenterally daily, and a fourth 2 per cent sulphetrone and 10 mg. of streptomycin. The control group of 11 animals remained untreated.

2. Twice during treatment all animals were tested for reactions to the intradermal injection of Old Tuberculin. There appeared to be a simple relation between the degree of severity of the intradermal reactions and the course of the disease judged by weight records and enlargement of lymph nodes.

3. Records of losses in weight, enlargement of glands, relation to tuberculin tests, macroscopic evidence of distribution of tuberculosis *post mortem*, and the evidence of histology, all presented a uniform picture of degrees of protection. All drug-treated groups showed evidence of protection. It was least in the promin group, became greater in the sulphetrone group, still greater in the streptomycin group, and was greatest in the group treated with sulphetrone and streptomycin. In the latter group the protection was so marked as to be clearly synergistic.

4. The opinion is expressed that the disease was progressive in all groups.

5. Strains of the tubercle bacillus recovered from the drug-treated groups were unchanged in their susceptibility to the antibacterial action of the drugs.

6. The experimental effects produced by combined streptomycin and sulphetrone therapy are believed to justify a careful clinical evaluation in selected cases.

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THE CHEMOTHERAPY OF AMOEBIASIS

PART I. INTRODUCTION AND METHODS OF BIOLOGICAL ASSAY

BY

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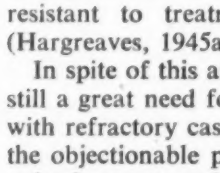
Three types of drug are commonly used for the treatment of amoebiasis: (*a*) emetine or its sparingly soluble derivatives such as the bismuth iodide (" E.B.I.") ; (*b*) halogenated quinolines such as 7-iodo-8-hydroxyquinoline-5-sulphonic acid (chiniofon B.P., " yatren," " quinoxyl "), 5-chloro-7-iodo-8-hydroxyquinoline (" vioform ") and 5 : 7-diiodoquinoline (" diodoquin ") ; and (*c*) arsenical preparations such as 4-carbamidophenylarsonic acid (carbarsone) and 3-acetamido-4-hydroxyphenylarsonic acid (acetarsone B.P.C., " stovarsol "). It is usual in current practice to administer courses of these drugs together with retention enemata of chiniofon. Recently penicillin and sulphonamides have also been given to control secondary infections and this treatment has been successful in a number of cases previously resistant to treatment with amoebicides alone (Hargreaves, 1945a and b ; 1946).

In spite of this armoury of medicaments there is still a great need for a new drug, not only to deal with refractory cases, but also if possible to avoid the objectionable properties of emetine. Ipecacuanha is scarce and expensive, and its most important alkaloid, emetine, although highly specific, is toxic and even in its pharmaceutically most elegant forms causes nausea and vomiting in many patients. The systematic search for new amoebicides has been hampered by the lack of a reliable *in vitro* test and of a convenient experimental infection in animals.

Pyman (1937a and b) examined a homologous series of harmol ethers (I) in which R was an alkyl group containing from two to twelve C atoms. A peak in amoebicidal activity



(I)

$(C_5H_{11})_2N–(CH_2)_{10}–N–(C_5H_{11})_2$

(II)

in vitro was found with $R = C_9H_{19}$ but the compounds were very sparingly soluble. In order to increase the solubility, basic groups were introduced into the terminal position of the alkyl groups [(I),$R = Alk_2N(CH_2)_x$] ; the number of methylene groups and the size of the alkyl groups were varied and a peak in activity was found in O-11-di-*n*-butylamino-undecylharmol [(I),$R = (C_4H_9)_2N(CH_2)_{11}$-] which, under the conditions of the test used, was lethal to *Entamoeba histolytica* in a dilution of 1 : 750,000 to 1 : 4,000,000. As this was many times more active than O-*n*-nonylharmol it was conjectured that the harmol residue might not play an important part in the amoebicidal action, and this part of the molecule was replaced by other groups, leading ultimately to the preparation of 1 : 10-bis-(di-*n*-amylamino)-decane (II) which was found to be three to five times as efficient as emetine. Unfortunately a clinical trial failed to confirm the promise of the *in vitro* test. It is evident from this careful work of Pyman and his collaborators that an *in vitro* test alone is not enough to indicate whether or not a compound is likely to be useful in the treatment of amoebiasis.

The present series of papers describes an attempt to synthesize amoebicidal substances based upon the structure of emetine, and upon the structure of certain compounds found by Pyman to be active *in vitro*. Details of the methods used for biological tests, and the results obtained with standard amoebicides will be described in the present paper, and an account of the new compounds prepared and their biological activities will be given in Parts II and III.

In Vitro Amoebicidal Tests

The activity of ipecacuanha alkaloids upon free-living amoebae was demonstrated by Vedder (1911) and by Pyman and Wenyon (1917), but satisfactory evidence of specific activity against *E. histolytica* was not forthcoming until a medium was devised which would maintain the amoebae alive sufficiently long for the drugs to take effect, and which contained no solid phase to remove the alkaloid

from solution. The buffered horse-serum Ringer medium (hs) of Laidlaw, Dobell, and Bishop (1928) met these requirements and was used by Pyman and his collaborators (1937a and b) for the examination of new compounds. A further source of difficulty has been the fact that so far it has not been possible to grow *E. histolytica* in the absence of bacteria, and the results of *in vitro* tests have been complicated by the presence of an indeterminate mixed bacterial flora used by the amoeba as a source of essential metabolites. Recent American work (Rees, Reardon, Jacobs, and Jones, 1941) has shown that it is possible to grow the organism in the presence of single strains of bacteria. In this country Dobell has succeeded in growing *E. histolytica in vitro* in the presence of a single pure strain of *Bact. coli*, and it was reported by Hargreaves (1945) that in such a culture the amoebae were killed by a concentration of emetine hydrochloride as low as 1 : 5,000,000. The use of this culture enables the observer to detect bactericidal action of a new compound which might be confused with direct amoebicidal action in a culture containing many strains of bacteria, some but not all of which were affected by the drug. The work of Pyman and the more recent work of Rawson and Hitchcock (1947) suffer from the disadvantages resulting from the presence of a mixed bacterial flora.

Methods used in the present work

For the cultures used, and for the method of test described below, we are indebted to Dr. Clifford Dobell. Two strains of amoeba have been used, both growing in culture with a single "smooth" strain of *Bact. coli*. One of them was obtained from a natural infection in a monkey, and was morphologically indistinguishable from *E. histolytica*; the other was isolated from man. These strains were identical in their reaction to emetine *in vitro*. The cultures were maintained on the horse-serum Ringer-egg (HSre) medium of Dobell and Laidlaw (1926). For an *in vitro* test, a series of 4 in.×1/2 in. tubes were prepared, each containing 4.5 ml. of buffered horse-serum—Ringer medium (hs) and 0.5 ml. of a sterile solution of drug in buffered Ringer. Each was inoculated with 6 drops (0.2 ml.) of a heavy culture of *Bact. coli* in Douglas-Hartley broth, and a large loopful (20 mg.) of dry, sterile rice starch added. The tubes were incubated overnight at 37° C. for growth of the bacteria to take place, and for the conditions at the base of each tube to become anaerobic. This may be confirmed in a sample tube by the addition of methylene blue before incubation; if growth is satisfactory the dye is bleached at the bottom of the tube. A heavy suspension of *E. histolytica* was then added to each tube, the inoculum being placed carefully on to the surface of the starch with a pipette. Large quantities of inoculum were obtained by culture in flasks of HSre medium prepared by the method of Frye and Meleney (1935). After incubation for three days, the tubes were examined microscopically for the growth of amoebae, the bacteria present tested for viability by subculture, and the *p*H of each tube of culture checked. The results obtained with standard amoebicides are recorded in Table I.

TABLE I

THE ACTIVITY OF AMOEBICIDAL DRUGS *in vitro*

Drug	Growth of amoebae in the presence of various concentrations of drug			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Emetine HCl ..	−	−	±	+
Carbarsone ..	±	+	+	+
Acetarsone	+	+	+	+
Chiniofon	−	+	+	+
Diodoquin* ..	±	+	+	+

*Almost insoluble; solid particles present.

None of these drugs had bactericidal activity at the dilutions used.

In Vivo Amoebicidal Tests

Still greater difficulties have been encountered in the finding of a suitable experimental infection upon which the *in vivo* activity of drugs could be investigated. Thousands of cats have been used in some laboratories (Wagner, 1935; Bieling, 1935; Kikuth, 1945) in spite of the fact that Dale and Dobell had shown in 1917 that the acute and lethal infection produced in kittens by the inoculation of *E. histolytica* per rectum was not affected by doses of emetine large enough to be toxic to the host, and that therefore experiments with new drugs could not be expected to yield much useful information.

Several workers have reported infection of rats and mice by the oral inoculation of *E. histolytica* cysts but such experiments have not been uniformly successful in the past. Meleney and Frye (1932) and Deschiens and Provost (1937) described the infection of cats by the direct inoculation of trophozoites into the lower ileum: in a recent paper by Jones (1946) this method has been modified and applied successfully to young rats, and a method of testing drugs has been carefully worked out. Our own experiments made independently during the past two years and described in the present series of papers produced similar results, showing that the young rat is a suitable host for caecal infections of *E. histolytica*. The infection in the rat caecum differs from that in the human

gut in that we have never yet seen an ulcer of the deep flask-shaped type in which amoebae are living exclusively upon the tissues of the host. The ulcers are extensive but shallow and rarely involve tissues deeper than the mucosa. The active amoebae frequently contain bacteria, whereas ingestion of red cells is only occasionally encountered, and cysts are rare. Nevertheless, as infected rats can be cured by the drugs used in human therapeutics, we consider the method to be the best so far devised for the investigation of new compounds designed as amoebicides.

Methods used in the present work

Animals

Very young rats weighing 20–30 g. were used immediately after weaning. At this age the animals have not developed an extensive caecal flora and fauna, having had milk as their main diet. The caecum is small and almost empty apart from a mucous secretion. At a later age the caecum becomes enlarged and distended with food residues and living organisms, and the inoculation of *E. histolytica* is less likely to produce an acute infection. Chronic light infections could be produced in older rats, but were less suitable for therapeutic experiments.

We were troubled in the early stages of this work by natural infections of the rats by an amoeba resembling *E. muris*. This organism is distinguishable from *E. histolytica* in stained smears, but is difficult to detect in fresh preparations especially when *E. histolytica* is also likely to be present. We also had the impression that a rat infected with *E. muris* was difficult to infect with *E. histolytica*, caecal conditions favourable to the former being apparently unsuitable for the latter. Experiments in these laboratories by Neal (1947) showed that hamsters frequently harboured heavy caecal infections of *E. muris* and discharged cysts which were infective to rats. They therefore served as a reservoir from which young rats readily became infected. Rats could be kept free from infection by keeping them in a room apart from hamsters and by bedding them in clean wood-wool sterilized by heating at 150° C. for 1 hour.

Strain of amoeba

We have tried a number of strains isolated from human patients, and find them to be variable in their pathogenicity to rats. It is possible that this is caused by differences in associated bacterial flora. The strain we have used for routine tests ("Strain B") was derived from a man infected in Burma, and had resisted a total of 11 courses of treatment with emetine and other drugs before it reached our hands. We are indebted to Air Commodore T. C. Morton for the material. This organism when grown in bulk upon HSre medium and inoculated intracaecally caused infection of 80 to 100 per cent of young rats. We did not find it necessary to add mucin to the inoculum as recommended by Jones (1946).

Method of inoculation

The rats were lightly anaesthetized with ether, the skin of the abdomen shaved, and the caecum exposed through a very small incision slightly to the left of the mid-line. A volume of 0.2 to 0.4 ml. of a rich suspension of amoebae from culture was injected directly into the caecum, the jet of fluid being directed in such a way that the blind end of the caecum was completely filled and good contact was made with the mucosa. A small amount of a mixture of equal parts of sulphanilamide and marfanil (about 10 mg.) was dusted into the abdominal wound to minimize chances of secondary infection, and the wound was closed with a rayon suture in the muscle and a Michel clip in the skin. The dusting with sulphonamides has no effect upon the development of the amoebic infection.

Treatment with drug

The rats were divided into groups and fed for six days upon diet containing the drug to be tested. The diet was a Coward stock diet, and the amount of drug incorporated was determined by the results of toxicity tests. The daily food consumption of a newly weaned rat is about 4 g. per day and the maximum concentration used for any drug was 0.5 per cent, representing a daily dose of about 800 mg. per kg. A disadvantage of the drug–diet technique is that the drug may be distasteful to the rats, and the food intake is then less than normal. This can readily be detected by observation of the food consumed and if a new drug shows promise as an amoebicide, more accurate treatment can be ensured by frequent dosage by catheter. The drug-diet method is convenient for "screening" tests and ensures continuous treatment with drugs. A group of untreated infected controls was used in every test to check the infectivity of the inoculum.

Assessment of results

For the assessment of the effect of a drug, the criterion we used was the presence or absence of amoebae in the caecum, as demonstrated by microscopical examination and by culture in HSre medium. Caecal contents showing plentiful amoebae under the microscope will not always grow out in culture if the concomitant bacterial flora is unsuitable, but culture will sometimes demonstrate the presence of an infection not detected microscopically, and is thus a useful supplement to direct examination. The condition of the caecum of each rat was also assessed by a "scoring" method similar to that described by Jones (1946). The presence of amoebae did not enter into our scores, which were awarded upon macroscopical evidence only. The conditions of the caecal wall (normal = 0; extensive ulceration = 4) was scored separately from the condition of the contents

(normal = 0; mucus only = 4). We have found that a drug may provide marked protection of the caecal wall and yet not remove the amoebae from the contents; another drug may remove the amoebae, but as a result of its irritant action the caecal wall will appear thickened and the contents fluid or mucoid. Such examples cannot be scored by Jones's method, and we consider that the alternative system described above gives a better analysis of the effects of a drug.

The results obtained with a series of standard amoebicides are shown in Table II.

TABLE II

THE ACTIVITY OF AMOEBICIDAL DRUGS *in vivo*

Drug	Concentration in diet %	Condition of caecum		Rats free from infection	
		Wall (mean score)	Contents (mean score)	Proportion	Per cent
Normal rats ..	—	0	0	—	—
Infected controls	—	3.0	2.8	30/156	19
Emetine HCl ..	0.002	0.2	1.0	16/18	89
	0.001	0.6	0.5	12/15	80
	0.0005	1.3	0.9	2/8	25
	0.00025	1.3	1.3	0/4	0
Emetine bismuth iodide	0.05	1.9	1.7	9/9	100
	0.02	1.5	2.8	26/29	90
	0.01	1.0	1.7	27/30	90
	0.005	1.7	1.9	15/17	88
	0.002	2.0	2.1	1/7	14
Carbarsone ..	0.4	0	0.3	6/6	100
	0.2	1.6	1.7	10/22	45
	0.1	2.1	2.1	3/8	38
Acetarsone ..	0.4	0	0.6	7/7	100
	0.2	1.0	1.2	12/23	52
	0.1	1.3	1.1	3/8	38
Chiniofon ..	0.2	0	0	8/8	100
	0.1	2.8	2.5	4/17	24
	0.05	3.6	2.6	0/7	0
Diodoquin ..	0.5	0	0	8/8	100
	0.2	3.0	2.9	4/12	33
	0.1	2.9	3.0	2/9	22

Discussion

The activities of drugs *in vitro* shown in Table I demonstrate the highly specific action of emetine. The limiting amoebicidal concentration of 10^{-6} to 10^{-7} is in agreement with the findings of Dobell (reported by Hargreaves, 1945; 1946). All the other drugs were much less active, or showed no activity at 10^{-4}. The results of the *in vivo* tests (Table II) also show emetine to be the most active drug tested in spite of the fact that the strain was derived from a case which had resisted repeated courses of this and other drugs. Emetine bismuth iodide showed activity commensurate with its alkaloidal content; the inorganic fraction appeared to have no influence upon the potency of the drug in the acute experimental infection. The results of giving emetine in the diet were more successful than those reported by Jones (1946) in which subcutaneous injection of six doses of 2 mg./kg., or oral administration of a single dose of 12 or 15 mg./kg. protected only 60 per cent or less of the animals.* A drug diet containing 0.001 per cent of emetine HCl, corresponding to a daily intake of approximately 1.5 mg./kg. was sufficient to protect 12/15 rats infected with strain "B," though we have evidence that strains derived from other sources may be less susceptible to emetine. Rats receiving drug diet containing 0.02 or 0.05 per cent of E.B.I. showed considerable irritation of the caeca, the walls appearing thickened and pale and the contents fluid. This is shown by the higher scores at these dose levels.

Comparison of Tables I and II shows that the drugs tested fall into the same order of relative activity by both *in vitro* and *in vivo* methods. Experiments with other drugs, which will be recorded in Parts II and III, have shown that this is not always the case. In the past it was a common practice to calculate the "chemotherapeutic index" of a drug from the ratio:

$$\frac{\text{"maximum tolerated dose"}}{\text{"minimum curative dose"}}$$

These terms have fallen into disfavour because their errors are large and indefinable and an attempt to provide a more accurate index has been made by expressing it as the ratio of the median lethal and median effective doses (Wien, 1946). Dr. J. W. Trevan, at the January, 1947, meeting of the British Pharmacological Society, pointed out that such a ratio was of little value if the dose–response curves were not parallel, and also that it did not express the relationship required—namely, a comparison of the safe dose with the curative dose. He suggested that a better figure would be given by the ratio of the "LD 0.1" and the "CD 99.9," which can be regarded as accurate substitutes for "maximum tolerated" and "minimum curative" doses. These figures may be determined from the dose–response curves by extrapolation or by calculation from the regression formulae.

In Table III this method has been applied to the amoebicides. Toxicity determinations were made upon rats of the same age and strain as those

* *Added in proof.* In a more recent paper Jones (1947) reports results from repeated oral administration of drugs which are very similar to those found by us.

used for amoebicidal tests, and the figures were obtained graphically. The total daily dose commonly employed in human therapeutics is also recorded, and will be seen to compare reasonably well with the daily intake required to cure rats.

TABLE III

Drug	Maximum clinical daily dose mg./kg.	Acute toxicity to young rats LD 0.1 mg./kg. orally	Amoebicidal activity CD 99.9 mg./kg./day in diet	Chemotherapeutic index LD 0.1 / CD 99.9
Emetine HCl ..	1	6	4.5	1.3
Emetine bismuth iodide ..	3	11	15	0.73
Carbarsone ..	14	4,500	600	7.5
Acetarsone ..	10	1,200	600	2
Chiniofon ..	30	800	300	2.7
Diodoquin ..	30	>40,000	600	>67

Table II shows that, in spite of their specific activity, it was difficult to obtain 100 per cent of cures with emetine HCl or emetine bismuth iodide. This was probably because emetine drug-diets containing more than about 0.001 per cent are irritant and distasteful to rats; the total dose therefore may not have been taken each day. On the other hand individual rats may carry infections which resist treatment with the alkaloid. In either event the question requires further investigation. The CD 99.9 values for these two drugs were estimated by extrapolation of the linear parts of the dose–response curves and are therefore probably too low. Reference to Table III shows that even these low figures are for emetine bismuth iodide within, and for emetine hydrochloride almost within, the toxic range. Chiniofon is the next most active, and also the next most toxic drug. Carbarsone appears to be better than acetarsone because of its lower toxicity, and diodoquin has the highest chemotherapeutic index of all because no rats died after an oral dose as high as 40 g./kg. Since it has no appreciable activity *in vitro* even when solid particles of drug are present (Table I), it is probable that diodoquin is converted to a more active substance during its passage through the body.

In clinical practice none of the drugs is successful in every case.

Summary

1. Methods are described for the comparison of the amoebicidal activities of drugs: (*a*) *in vitro*, using a strain of *Entamoeba histolytica* growing in the presence of a single strain of *Bacterium coli*: and (*b*) *in vivo*, using young rats inoculated intracaecally with cultures of amoebae.

2. The following standard amoebicides have been tested: emetine, emetine bismuth iodide, carbarsone, acetarsone, chiniofon, and diodoquin. Emetine was the most active, both *in vitro* and *in vivo*.

3. The chemotherapeutic indices of the drugs have been calculated and show that the curative dose of emetine is very close to the toxic dose. Of the drugs tested, diodoquin had the most favourable chemotherapeutic index.

Our thanks are due to Messrs. P. Amsden and J. M. Judd for valuable technical assistance.

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Brit. J. Pharmacol. (1948), **3**, 49.

THE CHEMOTHERAPY OF AMOEBIASIS

PART II. AMINES DERIVED FORMALLY FROM EMETINE

BY

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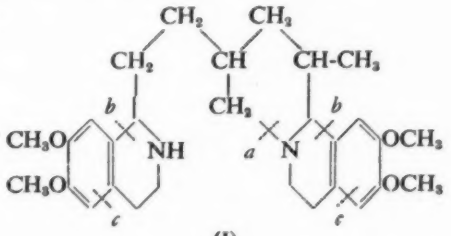
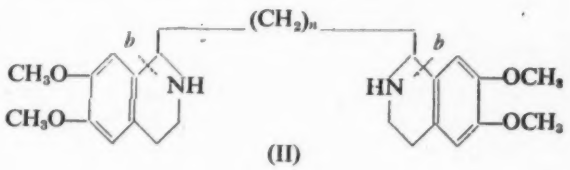
The structure of emetine according to Brindley and Pyman (1927) is probably represented by (I). If the bond shown by the dotted line at (*a*) in (I) is considered to be broken, a structure is derived consisting of two 6:7-dimethoxytetrahydro*iso*-quinoline nuclei joined at the 1 : 1′-positions by a chain of five methylene groups bearing two branches. Child and Pyman (1929) have synthesized a series of $\alpha\omega$-bis(6 : 7-dimethoxytetrahydro*iso*quinolyl) alkanes of formula (II) based on this model and found the compounds to be inactive against *Entamoeba histolytica in vitro*.



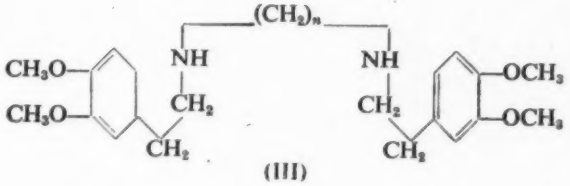
(I)

We have prepared a series of compounds (III ; $n=6$ to 10) modelled on the bis (β-3 : 4-dimethoxyphenylethylamino) alkanes resulting from the further rupture at (*b*) (*b*) of the ring system (II). The nature of the substituents in the benzene ring has been varied, and also the length of the central chain of methylene groups and of the chain between the amino group and the benzene ring.

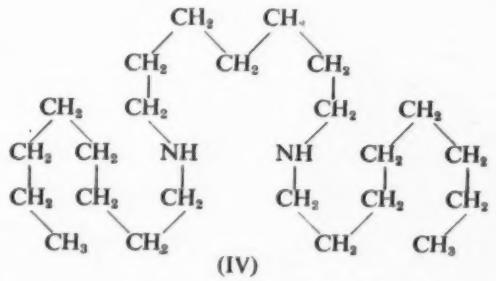
A series of bis(alkylamino)alkanes has also been prepared, the formal relationship of which to emetine is exemplified in bis(octylamino)heptane (IV). This structure is derived from that of emetine by opening out the molecule at the bonds (*a*), (*b*) (*b*) and (*c*) (*c*) in formula (I). In this series the length of the central chain of methylene groups has been varied and the chain branched, and the terminal alkyl groups have been varied in a similar manner.



(II)



(III)



(IV)

All these substances have been tested for amoebicidal activity by the methods outlined in the first paper of this series (Goodwin, Hoare and Sharp, 1948), and the results are shown in Table I. Selected members of the series have also been tested against experimental infections with trypanosomes, leishmania and malaria. The chemical syntheses and characteristics of the compounds are described in the chemical section on page 56.

As the quantities of drug available have in some cases been limited, the numbers of animals used were small. The results of the toxicity tests are very approximate, having been calculated by Kärber's method (Irwin and Cheeseman, 1939) from three or four groups of 5 mice. It will be

TABLE I

Columns 6 and 7: "+" signifies improvement, "—" no improvement.
Columns 10 and 11: "—" signifies no activity at a concentration of 10^{-4}.
Figures in parentheses denote slight activity with large doses. (e.g. "(6)" for compound No. 13 means that there was slight antimalarial activity at toxic dose levels.)

Column 12: "1" signifies no activity against *T. equiperdum*.
"2" " " " " *T. rhodesiense*.
"3" " " " " *T. congolense*.
"4" " " " " *T. cruzi*.
"5" " " " " *Leishmania donovani*.
"6" " " " " *Plasmodium gallinaceum*.

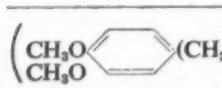
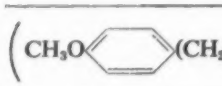
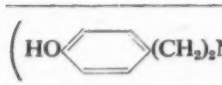
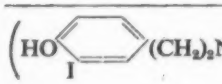
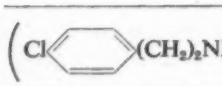
Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoebicidal conc.	Bact.-cidal conc.	
					Wall	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
 $(CH_2)_6$, 2HBr	1	950	250	0.2 0.1	+ —	+ —	4/7 2/8	60 25	—	—	1,3, 5,6.
,, $(CH_2)_6$, $2C_3H_6O_3$	2	..	..	0.5 0.05	 —	 —	5/6 1/6	80 15	..	..	..
,, $(CH_2)_7$, 2HBr	3	250	150	0.2 0.1	— —	— —	4/7 1/6	60 15	—	—	1,6.
,, $(CH_2)_8$, 2HBr	4	600	200	0.2 0.1 0.05	— — —	— — —	4/7 3/7 1/7	60 40 15	—	—	1,6.
,, $(CH_2)_9$, 2HBr	5	600	200	0.2 0.1 0.05	— — —	— — —	1/8 4/8 1/8	10 50 10	10^{-4}	—	6.
,, $(CH_2)_{10}$, 2HBr	6	700	550	0.2 0.1 0.05	+ + —	+ — —	3/4 2/7 1/7	75 30 15	10^{-5}	—	3,6.
 $(CH_2)_6$, 2HBr	7	..	..	0.2	—	—	0/4	0	10^{-4}	—	..
,, $(CH_2)_{10}$, 2HBr	8	30	40	0.1	—	—	0/4	0	—	—	1,3.
 $(CH_2)_6$, 2HCl	9	..	310	0.2	—	—	0/4	0	—	—	1,3.
,, $(CH_2)_{10}$, 2HCl	10	>2000	375	0.2	—	—	0/6	0	—	—	..
 $(CH_2)_6$	11	>2000	..	0.5	+	+	4/6	65	—	—	6.
,, $(CH_2)_{10}$	12	>2000	>2000	0.5	—	—	0/6	0	—	—	2,3.
 $(CH_2)_6$, 2HBr	13	>2000	>2000	0.2 0.1 0.05	+ — —	— — —	4/6 0/5 0/7	65 0 0	10^{-4}	10^{-4}	2,3. (6)
,, $(CH_2)_8$, 2HBr	14	>2000	>2000	0.2 0.1 0.05	+ + —	— — —	9/9 8/15 4/14	100 55 30	10^{-5}	—	..

TABLE I—*continued.*

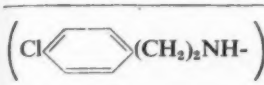
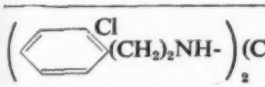
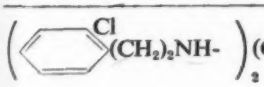
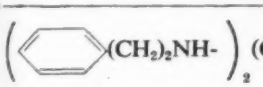
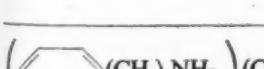
Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoebicidal conc.	Bact.-cidal conc.	
					Wall	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	15	450	>2000	0.5	Toxic				10^{-5}	—	6.
				0.2	—	—	Toxic				
				0.1	—	—	2/5	40			
,, Bismuth iodide (25.3% base)	16	..	..	0.5	+	+	5/6	80	..	..	..
				0.2	+	+	3/7	40			
	17	742	375	0.5	Toxic						
				0.2	+	+	6/6	100	10^{-6}	10^{-4}	..
				0.1	+	+	4/8	50			
,, Bismuth iodide (26% base)	18	..	..	0.5	+	+	4/6	65	..	..	..
				0.2	—	+	2/8	25			
	19	536	134	0.5	Toxic						
				0.2	+	+	3/4	75	10^{-5}	10^{-4}	6.
				0.1	—	+	1/7	15			
,, Bismuth iodide A (36.6% base)	20	..	..	0.5	+	+	5/5	100	..	..	..
				0.2	—	—	1/8	10			
,, Bismuth iodide B (22.9% base)	21	..	..	0.5	+	+	5/8	60	..	..	..
				0.2	—	—	0/8	0			
	22	120	350	0.5	—	—	Toxic		10^{-5}	—	(1),3,
				0.2	—	—	2/7	30			5.
,, ,, $2C_3H_6O_3$	23	..	..	0.2	—	—	0/3	0	..	..	1,3.
				0.1	—	—	1/6	20			
				0.05	—	—	2/6	30			
,, $(CH_2)_7$, 2HBr	24	165	400	0.5	Toxic						
				0.2	+	+	4/8	50	10^{-6}	—	(1).
				0.1	—	—	0/8	0			
				0.05	—	—	1/6	15			
,, $(CH_2)_8$, 2HBr	25	200	550	0.5	+	+	Toxic				
				0.2	+	+	9/12	75	10^{-5}	—	..
				0.1	—	—	2/7	30			
,, $(CH_2)_9$, 2HBr	26	135	68	0.5	+	+	Toxic				
				0.2	+	+	7/8	90	10^{-6}	—	
				0.1	—	—	7/8	90			..
				0.05	—	—	2/8	25			
,, $(CH_2)_{10}$, 2HBr	27	450	700	0.2	+	+	3/5	60			
				0.1	+	+	2/4	50	10^{-6}	—	1.
				0.05	—	—	3/5	60			
	28	710	410	0.5	+	+	Toxic		10^{-5}	—	..
				0.2	—	—	3/7	40			

TABLE I—*continued.*

Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoe-bicidal conc.	Bact.-cidal conc.	
					Wall	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
$\left(\text{C}_6\text{H}_4(\text{CH}_2)_3\text{NH-}\right)_2(\text{CH}_2)_{10}, 2\text{C}_3\text{H}_6\text{O}_3$	29	1400	700	0.5	Toxic				10^{-5}	—	..
				0.2	+	+	0/4	0			
$\left(\text{C}_6\text{H}_4(\text{CH}_2)_4\text{NH-}\right)_2(\text{CH}_2)_8, 2\text{C}_3\text{H}_6\text{O}_3$	30	308	310	0.2	—	—	1/6	15	10^{-5}	—	..
,, $(\text{CH}_2)_{10}, 2\text{C}_3\text{H}_6\text{O}_3$	31	>2000	>2000	0.5	+	+	Toxic		10^{-5}	—	..
				0.2	+	+	4/8	50			
$\left(\text{C}_6\text{H}_5(\text{CH}_2)_5\text{NH-}\right)_2(\text{CH}_2)_8, 2\text{C}_3\text{H}_6\text{O}_3$	32	..	..	0.2	—	—	3/7	40	10^{-5}	—	..
$\left(\text{C}_6\text{H}_4\text{CH}_2\text{NH-}\right)_2(\text{CH}_2)_8, 2\text{HCl}$	33	1400	350	0.5	—	—	3/7	40	..	..	..
				0.2	—	—	5/7	70			
$\left(\text{CH}_2\text{-O-C}_6\text{H}_4\text{CH}_2\text{NH-}\right)_2(\text{CH}_2)_8, 2\text{HCl}$	34	1400	1400	0.2	+	+	8/13	60	—	—	..
				0.1	+	+	3/7	45			
$\left(\text{(CH}_3\text{O)}_2\text{C}_6\text{H}_3\text{CH}_2\text{NH-}\right)_2(\text{CH}_2)_8, 2\text{HCl}$	35	..	..	0.5	—	—	2/8	25			
				0.2	—	—	0/7	0	10^{-4}	—	..
$\left(\text{CH}_3\text{O-C}_6\text{H}_4\text{CH}_2\text{NH-}\right)_2(\text{CH}_2)_8, 2\text{HCl}$	36	..	..	0.5	—	—	2/7	30			
				0.2	—	—	3/6	50	10^{-5}		
$\left(\text{(CH}_3\text{)}_2\text{N-C}_6\text{H}_4\text{CH}_2\text{NH-}\right)_2(\text{CH}_2)_8, 4\text{HCl}$	37	350	175	0.2	—	—	3/8	40	10^{-4}	—	..
$(\text{C}_7\text{H}_{15}\text{NH-})_2(\text{CH}_2)_6, 2\text{HBr}$	38	450	100	0.2	+	+	4/7	60	10^{-5}	10^{-4}	(1),3,
				0.1	+	+	4/10	40			5,6.
,, $2\text{C}_3\text{H}_6\text{O}_3$	39	..	154	0.5	+	+	5/5	100			(1),4,
				0.2	+	+	1/5	20	10^{-5}	10^{-4}	5,6.
				0.1	+	+	2/7	30			
$(\text{C}_7\text{H}_{15}\text{NH-})_2(\text{CH}_2)_7, 2\text{HBr}$	40	350	300	0.2	+	+	3/6	50	10^{-5}	10^{-4}	(1).
				0.1	—	—	5/15	33			
				0.05	—	—	4/9	40			
,, $(\text{CH}_2)_8, 2\text{HBr}$	41	450	350	0.2	+	+	4/8	50	10^{-5}	10^{-4}	1,6.
				0.1	+	+	3/5	60			
				0.05	+	+	1/4	25			
,, ,, $2\text{C}_3\text{H}_6\text{O}_3$	42	..	..	0.2	—	—	1/2	..	..	..	..
,, $(\text{CH}_2)_9, 2\text{HBr}$	43	450	450	0.2	Toxic						
				0.1	+	+	12/16	75	10^{-4}	—	6.
				0.05	—	—	3/12	25			
				0.025	—	—	5/11	45			
				0.0125	—	—	0/5	0			

TABLE I—*continued*

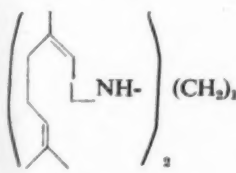
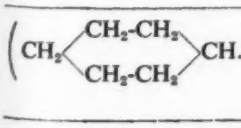
Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoe-bicidal conc.	Bact.-cidal conc.	
					Wall	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
$(C_7H_{15}NH\text{-})_2(CH_2)_{10}$, 2HBr	44	500	350	0.2	+	+	8/9	90			1,3,
				0.1	−	−	3/9	35	10^{-5}	10^{-4}	5,6.
				0.05	−	−	4/4	100			
				0.025	−	−	1/8	10			
				0.01	−	−	2/8	25			
$(C_6H_{13}NH\text{-})_2(CH_2)_{10}$, $2C_3H_6O_3$	45	1000	270	0.5	+	−	Toxic				
				0.2	−	−	Toxic		10^{-4}	−	..
				0.1	−	−	3/8	40			
$(C_8H_{17}NH\text{-})_2(CH_2)_8$, 2HBr	46	1800	>2000	0.2	+	+	11/14	80	10^{-5}	10^{-4}	1,3.
				0.1	−	−	1/6	15			
				0.05	−	−	7/23	35			
				0.01	−	−	6/14	40			
,, ,, $2C_3H_6O_3$	47	615	>2000	0.5	+	+	Toxic				
				0.2	+	+	4/4	100	..	..	..
				0.1	−	−	2/6	35			
$(C_9H_{19}NH\text{-})_2(CH_2)_8$, $2C_3H_6O_3$	48	700	>2000	0.2	+	+	Toxic				
				0.1	−	−	1/5	20	10^{-4}	−	..
$(C_{11}H_{23}NH)_2(CH_2)_{10}$, 2HBr	49	>2000	>2000	0.2	−	−	Toxic		−	−	
$(C_4H_9CH(C_2H_5)CH_2NH\text{-})_2(CH_2)_{10}$, 2HBr	50	>2000	>2000	0.2	−	−	0/3	0	10^{-5}	−	6.
$((CH_2{=}CH\text{-}CH_2)_3C.NH\text{-})_2(CH_2)_{10}$, 2HCl	51	1400	2000	0.5	+	+	Toxic				
				0.2	+	+	3/6	50	..	..	..
 $(CH_2)_{10}$, 2HBr	52	700	700	0.1	Toxic						
				0.05	+	+	5/7	70			
				0.025	−	−	1/7	15	10^{-5}	−	..
$(C_8H_{17}NH.CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}H\text{-})_2(CH_2)_6$, 2HBr	53	1400	700	0.2	Toxic						
				0.1	+	+	3/5	60	10^{-5}	−	..
$(C_8H_{17}NH.CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}H\text{-})_2(CH_2)_6$, 2HBr	54	>2000	1400	0.2	−	−	1/7	15	10^{-5}	−	..
 $(CH_2)_8$, 2HCl	55	390	200	0.5	Toxic						
				0.2	+	+	3/4	75	10^{-4}	10^{-4}	2,3,
				0.1	+	+	4/5	80			
,, $(CH_2)_{10}$, 2HCl	56	850	400	0.2	−	−	1/6	15	10^{-5}	−	2,3,6.

TABLE I—*continued.*

Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoe-bicidal conc.	Bact.-cidal conc.	
					Wall	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
$\left(O\langle \begin{matrix}CH_2\text{-}CH_2\\ CH_2\text{-}CH_2\end{matrix} \rangle N\text{-}\right)_2(CH_2)_{10}$, 2HCl	57	1600	180	0.5	–	–	2/4	50	–	–	..
				0.2	–	–	2/8	25			
$((C_2H_5)_2N(CH_2)_2NH\text{-})_2(CH_2)_{10}$, 4HBr	58	1200	300	0.2	–	–	1/6	15	–	–	(6).
$(C_8H_{17}NH\text{-})_2(CH_2)_5$, 2HBr	59	700	350	0.5	+	+	Toxic		10^{-5}	–	..
				0.2	+	+	5/7	70			
				0.1	+	+	1/8	10			
				0.05	–	–	0/8	0			
$(C_3H_7NH\text{-})_2(CH_2)_3$, 2HBr	60	374	2000	0.5	–	–	1/4	25	–	–	1,3.
				0.2	–	–	1/4	25	–	–	
$(C_7H_{15}NH\text{-})_2(CH_2)_3$, 2HBr	61	10	275	0.2	–	–	6/11	55	10^{-5}	10^{-4}	6.
				0.1	–	–	3/6	50			
$(C_{13}H_{27}NH\text{-})_2(CH_2)_3$, $2C_3H_6O_3$	62	>2000	>2000	0.5	–	–	4/7	60	–	–	6.
				0.2	–	–	0/5	0			
				0.1	–	–	1/5	20			
$NH_2(CH_2)_{10}NH_2$, 2HCl	63	1500	250	0.1	–	–	1/6	15	–	–	..
$((C_4H_9)_2N\text{-})_2(CH_2)_{10}$, $2C_4H_6O_4$	64	500	950	0.5	–	–	3/6	50	10^{-4}	–	..
$((C_5H_{11})_2N\text{-})_2(CH_2)_{10}$, 2HCl	65	..	..	0.5	+	+	3/7	40	10^{-5}	–	..
				0.2	+	+	7/14	50			
				0.1	–	–	3/12	25			
CH_3O-C$_6$H$_4$-$(CH_2)_3N\langle \begin{matrix}CH_2\text{-}CH_2\text{-}CH_2\\ CH_2\text{-}CH_2\text{-}CH_2\end{matrix}$, HBr	66	70	470	0.1	–	–	1/4	25	–	–	1,3.

noted that in some instances the oral toxicity of a substance is greater than the subcutaneous toxicity. This rather unusual finding is explained by the fact that most of the compounds are irritant, and whereas a localized necrosis is produced by subcutaneous injection, oral administration may result in severe and fatal damage to the walls of the alimentary tract, with secondary infection.

In the amoebicidal tests, the proportion of rats protected from infection has been expressed as a percentage, which facilitates comparison of the effects of one drug with another. As we are very conscious of the fact that percentages calculated from small numbers of animals have little meaning, the actual numbers of animals used are recorded, so that the true significance of the percentage figures may be apparent. It should be remembered that control animals may show an infection rate as low as 80 per cent, and a figure of 20 per cent protection may not indicate activity of a drug. Control animals were used in every test. The numbers of animals used were insufficient to give accurate comparisons between drugs, but were adequate to indicate whether or not a drug was likely to be of value in the treatment of amoebiasis. The effect upon the macroscopic appearance of the caecal wall and contents has been recorded here merely as a significant improvement (+) or no significant improvement (–); detailed figures are not given.

Discussion of the Results in Table I

Phenylalkylamine series

All the members of the series of bis(β-3 : 4-dimethoxyphenylethylamino)alkanes (Nos. 1 to 6) showed some activity *in vivo* but there was no significant difference between them. The improvement of the caecum produced by the compound containing 10 methylene groups suggests that it may have a slightly higher potency than the rest, and this is confirmed by the *in vitro* test.

When only one methoxy-group was present as in Nos. 7 and 8, the activity was reduced, and a similar effect was apparent in compounds 9 and 10 which contain free hydroxy-groups in the *para* positions of the benzene nuclei.

The introduction of iodine atoms into the benzene nucleus as in Nos. 11 and 12 gave rise to insoluble compounds with no increase in activity, although in this case the hexane- was more active than the decane-derivative.

The β-*p*-chlorophenylethylamino derivatives 13, 14, and 15 had some activity ; the corresponding *o*-chloro analogues (Nos. 17 and 19) were rather more active both *in vivo* and *in vitro*, but were also more toxic.

In the bis(β-phenylethylamino)alkane series (Nos. 22–27) all the compounds showed activity of a high order *in vitro*. The greatest *in vivo* activity was found in the higher members of the series. The effect of varying the length of the link between the phenyl and the amino groups is shown in Nos. 28 to 33. The optimum length seems to be a chain of two methylene groups. An increase in activity in the bis(phenylmethylamino)alkane series was found when a methylenedioxy-group was introduced into the 3 : 4- positions (No. 34), but a single *p*-methoxy or *p*-dimethylamino group was deleterious (Nos. 36 and 37).

Alkylamine series

The complete "opening out" of the emetine molecule leads to two *n*-octylamine residues connected through the N atoms by a chain of methylene groups. Owing to the ready availability of *n*-heptylamine *via* heptaldoxime we first studied the action of bis(*n*-heptylamino) alkanes. All these compounds (Nos. 38 to 44) had *in vitro* activities of the same order as the bis(β-phenylethylamino) alkane series, but in addition had bactericidal action, which probably complicated the results of the *in vitro* tests. *In vivo* these compounds were perhaps slightly more active than the phenylethylamino derivatives, but the results at low dose levels were erratic.

Alteration of the length of the alkyl chain as in Nos. 45 to 49 had little effect upon the activity, but the higher members had greater toxic effects when given in the diet than the lower members.

Branching the side-chain as in No. 50, 1 : 10-bis(2′-ethyl-*n*-hexylamino)decane and in No. 51, 1 : 10-bis(triallylcarbinamino) decane, where the chain is both branched and unsaturated, did not increase the *in vivo* activity, but the longer, branched, unsaturated compound, bis-geranylamino)decane (No. 52) again showed a high *in vivo* activity, accompanied however by an increased toxicity.

The introduction of methyl groups into the methylene chain connecting the two basic radicals (Nos. 53 and 54) had little effect.

When the alkyl chain was replaced by a *cyclo*hexyl radical (Nos. 55 and 56) the activity was not appreciably affected, but replacement of the alkylamino residues by tertiary basic groups as in 1 : 10-NN′-dimorpholyldecane (No. 57) resulted in complete loss of activity both *in vitro* and *in vivo*. The introduction of a basic side chain as in 1 : 10-bis(β-diethylaminoethylamino)decane (No. 58) had a similar effect.

Reduction in the number of methylene groups connecting the two secondary amino groups to five or three, even when the molecular weight was increased by the introduction of longer alkyl groups, caused almost complete loss of activity (Nos. 59 to 62).

The primary diamine, 1 : 10-diaminodecane (No. 63), was completely inactive and the ditertiary bases, 1 : 10-bis(dibutylamino)- and 1 : 10-bis(diamylamino)decane (Pyman, 1937 ; Nos. 64 and 65), were considerably less active than the disecondary bases of about the same molecular weight (*cf.* Nos. 44 and 47).

The cyclic tertiary amine No. 66, obtained as a by-product, was inactive.

The effect of solubility

The activities of the sparingly soluble hydrobromides and the readily soluble lactates were not significantly different (Nos. 1, 2 ; 22, 23 ; 38, 39 ; 41, 42 ; 46, 47). Nos. 15,17, and 19 have been converted into insoluble bismuth iodides of variable composition, Nos. 16, 18, 20 and 21 ; these were less toxic, but the activities were no greater than those of equivalent amounts of the parent compounds, except No. 16, which was slightly more active than No. 15.

None of the compounds is comparable with emetine in amoebicidal activity. Emetine protects most of the rats at a concentration in the diet of

0.001 per cent, whereas the best of the synthetic compounds are practically useless at concentrations lower than 0.1 per cent.

Although many members of the series have acute toxicities which are less than that of emetine, they are very irritant substances and it is unlikely that any would be suitable for clinical trial.

Chemical Section

Two general methods were used for the preparation of the secondary diamines; (a) an α,ω-alkylene dihalide was treated with a primary amine, or (b) an α,ω-alkylene diamine with an alkyl halide. The reactions were carried out in a variety of solvents—alcohol, amyl alcohol, ether, acetone, benzene, toluene or xylene—or without solvent, and for varying lengths of time. The choice of method was usually determined by the availability of the starting materials. In general the first method gave better yields, but, using alkylene dihalides containing 5 or 6 carbon atoms, there was a tendency for ring closure to take place with formation of N-alkyl(aralkyl)-piperidines or -hexamethyleneimines. The bis(phenylmethylamino) alkanes were most conveniently prepared *via* the anils.

1 : 6-*Bis*(β-3′ : 4′-*dimethoxyphenylethylamino*)*hexane* (No. 1). β-3 : 4-Dimethoxyphenylethylamine (7.24 g.), 1 : 6-dibromohexane (4.88 g.) and xylene (20 cc.) were allowed to stand for 24 hours and then heated to 115° for 30 mins. A crystalline solid separated on cooling; after recrystallization from alcohol glistening platelets of 1 : 6-*bis*(β-3′ : 4′-*dimethoxyphenylethylamino*)*hexane dihydrobromide*, m.p. 260–262°, were obtained in 29% yield. (Found: C, 51.5; H, 7.0; N, 4.6; Br, 26.6 $C_{26}H_{40}O_4N_2$,2HBr requires C, 51.5; H, 7.0; N, 4.6; Br, 26.4%.) The dilactate (No. 2) forms plates from acetone, m.p. 120°. (Found: C, 61.4; H, 8.4. $C_{26}H_{40}O_4N_2$,2$C_3H_6O_3$ requires C, 61.5; H, 8.4%.)

1 : 7-*Bis*(β-3′ : 4′-*dimethoxyphenylethylamino*)*heptane* (No. 3) was obtained similarly in 24% yield as *dihydrobromide* from 1 : 7-dibromoheptane and β-3 : 4-dimethoxyphenylethylamine. It forms leaflets from alcohol, m.p. 240°. (Found: N, 4.7; Br, 25.8. $C_{27}H_{42}O_4N_2$, 2HBr requires N, 4.5; Br, 25.8%.) The *dihydrochloride* forms needles from alcohol, m.p. 247°. (Found: C, 60.7; H, 8.2. $C_{27}H_{42}O_4N_2$,2HCl requires C, 61.0; H, 8.3%.)

1 : 8-*Bis*(β-3′ : 4′-*dimethoxyphenylethylamino*)*octane* (No. 4). The *dihydrobromide*, m.p. 251°, was obtained similarly from 1 : 8-dibromo-octane and the amine in 40% yield. (Found: N, 4.8; Br, 25.5. $C_{28}H_{44}O_4N_2$,2HBr requires N, 4.4; Br, 25.2%.) The *dihydrochloride* forms needles from alcohol, m.p. 245–247°. (Found: C, 61.8; H, 8.6; N, 5.3; Cl, 12.4. $C_{28}H_{44}O_4N_2$,2HCl requires C, 61.6; H, 8.5; N, 5.1; Cl, 13.0%.)

1 : 9 *Bis*(β-3′ : 4′-*dimethoxyphenylethylamino*)*nonane* (No. 5). The *dihydrobromide*, obtained similarly from 1 : 9-dibromononane in 32% yield, forms leaflets from alcohol, m.p. 248°. (Found: N, 4.6; Br, 24.7. $C_{29}H_{46}O_4N_2$,2HBr requires N, 4.3; Br, 24.65%.) The *dihydrochloride* forms needles from alcohol, m.p. 251°. (Found: Cl, 12.8. $C_{29}H_{46}O_4N_2$,2HCl requires Cl, 12.7%.)

1 : 10-*Bis*(β-3′ : 4′-*dimethoxyphenylethylamino*)*decane* (No. 6). The *dihydrobromide*, leaflets from alcohol, m.p. 251°, was obtained similarly in 45% yield from 1 : 10-dibromodecane and homoveratrylamine. (Found: N, 4.5; Br, 24.2. $C_{30}H_{48}O_4N_2$,2HBr requires N, 4.2; Br, 24.1%.) The *dihydrochloride* forms needles from alcohol, m.p. 244–245°. (Found: C, 62.9; H, 8.9. $C_{30}H_{48}O_4N_2$,2HCl requires C, 62.8; H, 8.8%.)

1 : 6-*Bis*(β-p-*methoxyphenylethylamino*)*hexane* (No. 7). β-*p*-Methoxyphenylethylamine (15.1 g.), alcohol (25 cc.) and 1 : 6-dibromohexane (6.1 g.) were heated under reflux for 24 hours and 2*N*-alcoholic HBr (25 cc.) added. The crystalline precipitate was filtered off and boiled with alcohol to remove *p*-methoxyphenylethylamine hydrobromide. The residue, recrystallized from a large volume of alcohol, deposited 1 : 6-*bis*(β-p-*methoxyphenylethylamino*)*hexane dihydrobromide* in crystals m.p. 308°. (Found: C, 52.8; H, 7.4; N, 5.1; Br, 29.1. $C_{24}H_{36}O_2N_2$, 2HBr requires C, 52.75; H, 7.0; N, 5.1; Br, 29.25%.) The *dipicrate* forms yellow crystals from alcohol, m.p. 198°. (Found: C, 51.3; H, 5.1; N, 13.5. $C_{24}H_{36}O_2N_2$, $2C_6H_3O_7N_3$ requires C, 51.3; H, 5.0; N, 13.3%.) The bases regenerated from the original mother-liquors were dissolved in alcohol and on addition of a hot alcoholic solution of picric acid deposited an oil; the clear decanted liquor on standing deposited orange crystals of β-*p*-methoxyphenylethylamine picrate mixed with yellow crystals of *N*-β-p-*methoxyphenylethylhexamethyleneimine picrate*. These were readily separated by hot benzene in which the former is insoluble. The latter formed yellow crystals from benzene, m.p. 151°, yield 37%. (Found: C, 54.7; H, 6.0; N, 12.2. $C_{15}H_{23}ON$,$C_6H_2O_7N_3$ requires C, 54.5; H, 5.7; N, 12.1%.) The *hydrobromide* (No. 66), colourless crystals from alcohol, melts at 196°. (Found: C, 57.3; H, 7.9; N, 4.4; Br, 25.6. $C_{15}H_{23}ON$, HBr requires C, 57.3; H, 7.9; N, 4.5; Br, 25.4%.) The ring structure was confirmed by failure of the base to yield an acetyl derivative and by the formation of a *methiodide*, m.p. 132°. (Found: C, 51.1; H, 7.1; N, 3.9; OMe, 8.3; NMe. 7.5. $C_{15}H_{23}ON$,CH_3I requires C, 51.2; H, 7.0; N, 3.7; OMe, 8.3; NMe, 7.7%.)

1 : 10-*Bis*(β-p-*methoxyphenylethylamino*)*decane* (No. 8). The *dihydrobromide* was obtained in 53% yield in a similar manner to that just described but omitting the treatment with picric acid. It separates from a large volume of alcohol in crystals, m.p. 304° (decomp.). (Found: N, 4.9; Br, 26.7. $C_{28}H_{44}O_2N_2$,2HBr requires N, 4.65; Br, 26.5%.)

1 : 6-*Bis*(β-p-*hydroxyphenylethylamino*)*hexane* (No. 9). This was prepared by heating the methoxy-derivative (No. 7) with 8 times its weight of hydrochloric acid (*d*, 1.2) in a sealed tube at 170° for 6 hours. The *dihydrochloride* separated from water in silky needles, m.p. 255–257°. (Found: C, 61.2; H, 7.9; N, 6.3; Cl, 16.8. $C_{22}H_{32}O_2N_2$,2HCl requires C, 61.5; H, 8.0; N, 6.5; Cl, 16.5%.)

1 : 10-*Bis*(β-p-*hydroxyphenylethylamino*)*decane* (No. 10). The corresponding methoxy-compound (No. 8) heated in a sealed tube with hydrochloric acid (*d*, 1.2) furnished 1 : 10-*bis*(β-p-*hydroxyphenylethylamino*)*decane dihydrochloride* in needles from water. m.p. 238° (decomp.).

(Found: C, 64.8; H, 8.7; N, 5.4; Cl, 14.7. $C_{26}H_{40}O_2N_2$, 2HCl requires C, 64.3; H, 8.7; N, 5.8; Cl, 14.6%.)

1 : 6-*Bis*(β-3′-*iodo*-4′-*hydroxyphenylethylamino*)*hexane* (No. 11). To a stirred solution of the hydroxy-compound (No. 9) (2.0 g.) in methyl alcohol (120 cc.) 10% aqueous ammonia (4 cc.) was added followed by iodine (2.4 g.) in 50% potassium iodide solution (9.6 g.). The precipitate was collected, washed in turn with methanol, water and methanol. The base formed an almost white powder, m.p. 175°, yield 94%. (Found: I, 42.4. $C_{22}H_{30}O_2N_2I_2$ requires I, 41.7%.) The iodine atoms are assumed to be in the 3′-positions.

1 : 10-*Bis*(β-3′-*iodo*-4′-*hydroxyphenylethylamino*)*decane* (No. 12). This was prepared in 93% yield from No. 10 in a similar way. The base melted at 169° (decomp.). (Found: C, 46.9; H, 6.2; N, 4.5; I, 40.3. $C_{26}H_{38}O_2N_2I_2$ requires C, 47.0; H, 5.8; N, 4.2; I, 38.2%.) The iodine atoms are assumed to be in the 3′-positions.

β-p-*Chlorophenylethylamine*. Dry ammonia was passed for 3–4 hours through fused *p*-chlorophenylpropionic acid at 180°. The powdered product was freed from a little unchanged acid by extraction with aqueous ammonia and the insoluble p-*chlorophenylpropionamide* recrystallized from benzene. It formed tetragonal plates, m.p. 133°, yield 82%. (Found: C, 59.3; H, 5.8; N, 7.9; Cl, 19.3. $C_9H_{10}ONCl$ requires C, 58.9; H, 5.5; N, 7.6; Cl, 19.3%.) It was converted into *p*-chlorophenylethylamine, b.p. 129–135/24 mm., by the method of McRae and Vining (1932). The *picrate* forms light orange-coloured prisms from alcohol, m.p. 212°. (Found: N, 14.8; Cl, 9.3. $C_8H_{10}NCl,C_6H_3O_7N_3$ requires N, 14.6; Cl, 9.2%.) The *hydrobromide* separates from alcohol in colourless crystals, m.p. 237°. (Found: C, 40.6; H, 5.0; N, 5.7. $C_8H_{10}NCl,HBr$ requires C, 40.6; H, 4.7; N, 5.9%.)

β-o-*Chlorophenylethylamine*. This base was prepared in 67% yield from β-*o*-chloropropionamide in a similar manner. It is a colourless liquid, b.p. 128°/23–26 mm. The *picrate* forms yellow crystals from alcohol, m.p. 187°. (Found: C, 43.7; H, 3.9; Cl, 9.2. $C_8H_{10}NCl,C_6H_3O_7N_3$ requires C, 43.7; H, 3.4; Cl, 9.2%.) The *hydrobromide*, which is very soluble in alcohol, separates from a concentrated solution as a *dihydrate*, m.p. 92° with previous sintering. (Found: loss at 50° in a vacuum 12.2. $C_8H_{10}NCl,2H_2O$ requires loss 13.2%.) The anhydrous salt has m.p. 190°. (Found: C, 40.9; H, 4.9; N, 6.1; Cl, 14.7; Br, 33.2. $C_8H_{10}NCl,HBr$ requires C, 40.6; H, 4.7; N, 5.9; Cl, 15.0; Br, 33.8%.)

1 : 6-*Bis*(β-p-*chlorophenylethylamino*)*hexane* (No. 13). β-p-Chlorophenylethylamine (4 mols.) in alcohol was heated under reflux for 24 hours with 1 : 6-dibromohexane (1 mol.). The product was neutralized with 2*N*-alcoholic hydrobromic acid and after standing the crystalline deposit was filtered off and recrystallized to give 1 : 6-*bis*(β-p-*chlorophenylethylamino*)*hexane dihydrobromide* in colourless crystals, m.p. 301° (decomp.), yield 29%. (Found: N, 5.2; Cl, 12.5; Br, 28.1. $C_{22}H_{30}N_2Cl_2,2HBr$ requires N, 5.1; Cl, 12.8; Br, 28.8%.) The *dipicrate* separated from alcohol in yellow crystals, m.p. 219°. (Found: C, 48.5; H, 4.8; N, 12.9; Cl, 8.0. $C_{22}H_{30}N_2Cl_2,2C_6H_3O_7N_3$ requires C, 48.0; H, 4.3; N, 13.2; Cl, 8.3%.) The bases liberated from the mother-liquors from the above hydrobromide were converted to picrates from which *N*-β-p-*chlorophenylethylhexamethylene*imine *picrate* was obtained by extraction with benzene. It separated from this solvent in yellow needles, m.p. 181° in a yield of 32%. (Found: C, 51.8; H, 5.4; N, 12.1; Cl, 7.5. $C_{14}H_{20}NCl,C_6H_3O_7N_3$ requires C, 51.45; H, 5.0; N, 12.0; Cl, 7.6%.) The *hydrobromide* forms plates from alcohol, m.p. 263° (decomp.). (Found: C, 52.8; H, 6.7; N, 4.6; Cl, 11.1; Br, 25.1. $C_{14}H_{20}NCl,HBr$ requires C, 52.75; H, 6.6; Cl, 11.1; Br, 25.1%.)

1 : 8-*Bis*(β-p-*chlorophenylethylamino*)*octane* (No. 14). The *dihydrobromide* was obtained in 44% yield by the above process using 1 : 8-dibromo-octane and *p*-chlorophenylethylamine. It is very sparingly soluble in alcohol from which it is deposited in crystals, m.p. 291° (decomp.). (Found: C, 49.4; H, 6.5; N, 4.9; Cl, 11.7; Br, 26.4. $C_{24}H_{34}N_2Cl_2,2HBr$ requires C, 49.4; H, 6.2; N, 4.8; Cl, 12.2; Br, 27.4%.) The *dipicrate* forms yellow crystals from alcohol, m.p. 183°. (Found : C, 49.3; H, 4.6; N, 12.6. $C_{24}H_{34}N_2Cl_2,2C_6H_3O_7N_3$ requires C, 49.2; H, 4.6; N, 12.7%.)

1 : 10-*Bis*(β-p-*chlorophenylethylamino*)*decane* (No. 15). The *dihydrobromide* was obtained in 53% yield from 1 : 10-dibromodecane and the appropriate amine in crystals from alcohol, m.p. 290° (decomp.). (Found: C, 51.3; H, 6.6; N, 4.6. $C_{26}H_{38}N_2Cl_2,2HBr$ requires C, 51.1; H, 6.6; N, 4.6%.) The *dipicrate* forms yellow needles from alcohol, m.p. 170°. (Found: C, 50.3; H, 5.0; N, 12.0. $C_{26}H_{38}N_2Cl_2,2C_6H_3O_7N_3$ requires C, 50.3; H, 4.9; N, 12.35%.) The *dilactate* separates from alcohol in crystals, m.p. 160°. (Found: N, 4.6; Cl, 11.9. $C_{26}H_{38}N_2Cl_2,2C_3H_6O_3$ requires N, 4.45; Cl, 11.3%.) The bismuth iodide (No. 16), formed by addition of excess of potassium bismuth iodide to a solution of the lactate in water forms an insoluble red powder approximating in composition to $B,2HI,BiI_3$. (Found: base 25.3. $B,2HI,BiI_3$ requires base, 23.8%.)

1 : 8-*Bis*(β-o-*chlorophenylethylamino*)*octane* (No. 17). The *dihydrobromide* was obtained in 38% yield by heating β-*o*-chlorophenylethylamine and 1 : 8-dibromo-octane in alcoholic solution. It is very sparingly soluble in alcohol from which it separates in crystals, m.p. 266° (decomp.). (Found: C, 49.8; H, 6.4; N, 4.7. $C_{24}H_{34}N_2Cl_2,2HBr$ requires C, 49.4; H, 6.2; N, 4.8%.) The *dipicrate* forms yellow crystals from alcohol, m.p. 154°. (Found: C, 49.3; H, 4.7; N, 13.1. $C_{24}H_{34}N_2Cl_2,2C_6H_3O_7N_3$ requires C, 49.2; H, 4.6; N, 12.7%.) The *dilactate* is very soluble in alcohol and crystallizes on addition of acetone, m.p. 171°. (Found: N, 4.6; Cl, 11.8. $C_{24}H_{34}N_2Cl_2,2C_3H_6O_3$ requires N, 4.65; Cl, 11.8%.) The bismuth iodide (No. 18) prepared in the usual manner from the lactate formed an insoluble red powder containing 26% of base.

1 : 10-*Bis*(β-o-*chlorophenylethylamino*)*decane* (No. 19). The *dihydrobromide* was obtained in 45% yield from 1 : 10-dibromodecane and the appropriate amine; it separates from much alcohol in crystals, m.p. 262° (decomp.). (Found: C, 51.4; H, 6.9; N, 4.3; Cl, 11.7; Br, 26.4. $C_{26}H_{38}N_2Cl_2,2HBr$ requires C, 51.1; H, 6.6;

N, 4.6; Cl, 11.6; Br, 24.15%.) The *dipicrate* forms yellow crystals from alcohol, m.p. 121°. (Found: N, 12.4; Cl, 7.5; $C_{26}H_{38}N_2Cl_2,2C_6H_3O_7N_3$ requires N, 12.35; Cl, 7.8%.) The *dilactate* (No. 19) is deposited from hot alcohol in crystals, m.p. 131°. (Found: C, 61.1; H, 8.1; N, 4.7. $C_{26}H_{38}N_2Cl_2,2C_3H_6O_3$ requires C, 61.0; H, 8.0; N, 4.45%.) Two different bismuth iodides were obtained by the addition of varying amounts of potassium bismuth iodide to an aqueous solution of the lactate. Preparation A (No. 20) contained 36.6% of base and B (No. 21) 22.9% of base. These correspond approximately to compounds of the composition $B,2HI,BiI_3$ and $B,2HI,2BiI_3$ which require 34.65% and 23.8% of base respectively.

1 : 6-*Bis*(β-*phenylethylamino*)*hexane* (No. 22). β-Phenylethylamine (10 g.) and 1 : 6-dibromohexane (10.16 g.) in benzene (30 cc.) were heated under reflux for 2 hours. After cooling 1 : 6-*bis*(β-*phenylethylamino*)-*hexane dihydrobromide* was collected and recrystallized from alcohol. It forms plates, m.p. 314–317°, yield 12.5%. (Found: C, 54.7; H, 7.2; N, 6.1; Br, 32.55. $C_{22}H_{32}N_2,2HBr$ requires C, 54.3; H, 7.05; N, 5.8; Br, 32.9%.) The *dilactate* (No. 23) separates from alcohol-acetone in prisms, m.p. 155–157°. (Found: C, 66.6; H, 8.95. $C_{22}H_{32}N_2,2C_3H_6O_3$ requires C, 66.7; H, 8.8%.)

1 : 7-*Bis*(β-*phenylethylamino*)*heptane* (No. 24). The *dihydrobromide* was obtained in 24% yield from 1 : 7-dibromoheptane and β-phenylethylamine by the above method. It forms plates from alcohol, m.p. 313°. (Found: C, 55.2; H, 7.4. $C_{23}H_{34}N_2,2HBr$ requires C, 55.2; H, 7.2%.) The *dilactate* separates from acetone in crystals, m.p. 150–152°. (Found: C, 67.3; H, 9.1. $C_{23}H_{34}N_2,2C_3H_6O_3$ requires C, 67.2; H, 8.9%.)

1 : 8-*Bis*(β-*phenylethylamino*)*octane* (No. 25). 1 : 8-Diamino-octane (4.32 g.), obtained by the action of hydrazoic acid on sebacic acid, was heated on a water bath for 15 mins. with β-phenylethyl bromide (11.1 g.) and benzene (10 cc.). The solid which separated was collected and crystallized from alcohol. The *hydrobromide* was thus obtained in leaflets, m.p. 312°, yield 27%. (Found: C, 56.4; H, 7.6. $C_{24}H_{36}N_2,2HBr$ requires C, 56.0; H, 7.45%.) The *dilactate* forms needles, m.p. 150–151°. (Found: C, 67.8; H, 9.1. $C_{24}H_{36}N_2,2C_3H_6O_3$ requires C, 67.6; H, 9.1%.)

1 : 9-*Bis*(β-*phenylethylamino*)*nonane* (No. 26). β-Phenylethylamine (12.1 g.) and 1 : 9-dibromononane (14.3 g.) dissolved in benzene (25 cc.) were left to stand at room temperature for 48 hours. The crystalline solid which separated was recrystallized from alcohol to give the *dihydrobromide*, m.p. 300–302°, in 21.6% yield. (Found: C, 56.8; H, 8.05. $C_{25}H_{38}N_2,2HBr$ requires C, 56.8; H, 7.7%.) The *dilactate* forms clusters of needles from alcohol-acetone, m.p. 134–136°. (Found: C, 68.3; H, 9.4. $C_{25}H_{38}N_2,2C_3H_6O_3$ requires C, 68.1; H, 9.2%.)

1 : 10-*Bis*(β-*phenylethylamino*)*decane* (No. 27). β-Phenylethylamine and 1 : 10-dibromodecane heated for 2 hours under reflux in benzene solution furnished the *dihydrobromide* in 13.5% yield after crystallization from alcohol, m.p. 302°–304°. (Found: C, 57.7; H, 7.9. $C_{26}H_{40}N_2,2HBr$ requires C, 57.6; H, 7.8%.) The *dilactate* forms needles from alcohol-acetone, m.p. 146°. (Found: C, 68.7; H, 9.1. $C_{26}H_{40}N_2,2C_3H_6O_3$ requires C, 68.5; H, 9.35%.)

1 : 8-*Bis*(γ-*phenylpropylamino*)*octane* (No. 28). The *dihydrobromide* was obtained in 37% yield by heating γ-phenylpropylamine (4 mols.) with 1 : 8-dibromo-octane (1 mol.) in alcoholic solution for 24 hours. It forms crystals sparingly soluble in alcohol, m.p. 284° (decomp.). (Found: C, 57.5; H, 8.2; N, 5.1; Br, 29.9. $C_{26}H_{40}N_2,2HBr$ requires C, 57.6; H, 7.8; N, 5.2; Br, 29.5%.) The *dipicrate* gave yellow crystals from alcohol, m.p. 153°. (Found: C, 54.3; H, 5.75; N, 13.7. $C_{26}H_{40}N_2,2C_6H_3O_7N_3$ requires C, 54.2; H, 5.5; N, 13.4%.) The *dilactate* crystallizes from alcohol-acetone, m.p. 147°. (Found: C, 68.5; H, 9.4; N, 5.5. $C_{26}H_{40}N_2,2C_3H_6O_3$ requires C, 68.5; H, 9.35; N, 5.0%.)

1 : 10-*Bis*(γ-*phenylpropylamino*)*decane* (No. 29). The *dihydrobromide*, obtained in a similar manner in 50% yield from 1 : 10-dibromodecane and the amine, is sparingly soluble in alcohol and forms crystals, m.p. 283°. (Found: C, 59.1; H, 8.2; N, 4.3; Br, 28.4. $C_{28}H_{44}N_2$, 2HBr requires C, 58.9; H, 8.1; N, 4.9; Br, 28.0%.) The *dilactate* separates from alcohol-acetone in crystals, m.p. 119°. (Found: N, 4.8. $C_{28}H_{44}N_2,2C_3H_6O_3$ requires N, 4.8%.)

1 : 8-*Bis*(δ-*phenylbutylamino*)*octane* (No. 30). The *dihydrobromide* was obtained similarly in 32% yield from δ-phenylbutylamine and 1 : 8-dibromo-octane. It crystallizes from alcohol in needles, m.p. 281° (decomp.). (Found: C, 58.3; H, 8.0; N, 5.3; Br, 28.9. $C_{28}H_{44}N_2$, 2HBr requires C, 58.9; H, 8.1; N, 4.9; Br, 28.0%.) The *dilactate* crystallizes on addition of acetone to a concentrated alcoholic solution, m.p. 149°. (Found: C, 69.5; H, 9.4; N, 5.0. $C_{28}H_{44}N_2,2C_3H_6O_3$ requires C, 69.35; H, 9.6; N, 4.8%.)

1 : 10-*Bis*(δ-*phenylbutylamino*)*decane* (No. 31). The *dihydrobromide* was obtained in 43% yield from δ-phenylbutylamine and 1 : 10-dibromodecane. It is sparingly soluble in alcohol, m.p. 282° (decomp.). (Found: C, 60.4; H, 8.2; N, 4.8; Br, 26.9. $C_{30}H_{48}N_2,2HBr$ requires C, 60.2; H, 8.4; N, 4.7; Br, 26.7%.) The *dilactate* crystallized from alcohol, m.p. 125°. (Found: C, 69.8; H, 9.4; N, 4.8. $C_{30}H_{48}N_2,2C_3H_6O_3$ requires C, 70.1; H, 9.8; N, 4.5%.)

1 : 8-*Bis*(ε-*phenylamylamino*)*octane* (No. 32). The *dihydrobromide* was obtained in 73% yield from ε-phenylamylamine and 1 : 8-dibromo-octane. It is fairly soluble in hot alcohol and separates in crystals, m.p. 258°. (Found: C, 60.5; H, 8.0; N, 4.5. $C_{30}H_{48}N_2,2HBr$ requires C, 60.2; H, 8.4; N, 4.7%.) The *dilactate* forms six-sided plates from alcohol-acetone, m.p. 117°. (Found: C, 70.0; H, 9.8; N, 4.7. $C_{30}H_{48}N_2,2C_3H_6O_3$ requires C, 70.1; H, 9.8; N, 4.5%.)

1 : 8-*Bis*(*phenylmethylamino*)*octane* (No. 33). 1 : 8-Diamino-octane (2.9 g.) was heated under reflux with benzaldehyde (4.5 g.) for 1 hour to yield 1 : 8-*bis*-(*benzylideneamino*)*octane* which after distillation at 220–230/0.1 mm. crystallized on cooling, m.p. 30–31°. (Found : C, 82.8; H, 8.8. $C_{22}H_{28}N_2$ requires C, 82.5; H, 8.8%.) The anil was reduced with hydrogen (PtO_2

catalyst) in alcoholic solution. On addition of hydrochloric acid (*d* 1.2) to the filtered solution 1:8-*bis*(*phenylmethylamino*)*octane dihydrochloride* was obtained m.p. 276–280° after crystallization from alcohol. (Found: C,66.4; H, 8.5; Cl, 17.9. $C_{22}H_{32}N_2$,2HCl requires C, 66.5; H, 8.6; Cl, 17.9%.) The *base* formed crystals, m.p. 37–38.5°. (Found: C, 81.1; H, 9.8; N, 8.5. $C_{22}H_{32}N_2$ requires C, 81.4; H, 9.9; N, 8.6%.)

1 : 8-*Bis*(3′ : 4′-*methylenedioxyphenylmethylamino*)*octane* (No. 34). By a similar procedure, piperonal was converted to 1:8-*bis*(3′:4′-*methylenedioxybenzylideneamino*)*octane*, m.p. 111–112° from alcohol. (Found: C, 70.8; H, 7.2. $C_{24}H_{28}O_4N_2$ requires C, 70.6; H, 6.9%.) Catalytic reduction gave the *base*, leaflets from alcohol, m.p. 69–70°. (Found: C, 69.7; H, 8.1; N, 6.6. $C_{24}H_{32}O_4N_2$ requires C, 69.9; H, 7.8; N, 6.8%.) The *dihydrochloride* melted at *ca.* 274°. (Found: C, 59.2; H, 6.9. $C_{24}H_{32}O_4N_2$,2HCl requires C, 59.4; H, 7.1%.)

1 : 8-*Bis*(3′ : 4′-*dimethoxyphenylmethylamino*)*octane* (No. 35). Veratric aldehyde was converted similarly into 1:8-*bis*(3′:4′-*dimethoxyberzylideneamino*)*octane*, leaflets from alcohol, m.p. 108.5–109.5°. (Found: C, 71.2; H, 8.5. $C_{26}H_{36}O_4N_2$ requires C, 70.9; H, 8.2%.)

The *base*, obtained by catalytic reduction of the anil, formed leaflets from alcohol, m.p. 83°. (Found: C, 69.9; H, 9.0. $C_{26}H_{40}O_4N_2$ requires C, 70.2; H, 9.1%.) The *dihydrochloride* crystallized from alcohol, m.p. 231–232° (decomp.). (Found: C, 60.5; H, 8.1; Cl, 14.0. $C_{26}H_{40}O_4N_2$,2HCl requires C, 60.4; H, 8.2; Cl, 13.7%.)

1 : 8-*Bis*(p-*methoxyphenylmethylamino*)*octane* (No. 36). *p*-Methoxybenzaldehyde with 1 : 8-diamino-octane yielded 1:8-*bis*(p-*methoxybenzylideneamino*)*octane*, leaflets from alcohol, m.p. 64–65°. (Found: C, 74.7; H, 8.3; N, 7.4. $C_{24}H_{32}O_2N_2$ requires C, 75.7; H, 8.5; N, 7.4%.) Reduction of the anil gave the *base*, m.p. 60.5–61.5°, after crystallization from light petroleum. (Found: C, 74.7; H, 9.4; N, 7.1. $C_{24}H_{36}O_2N_2$ requires C, 75.0; H, 9.4; N, 7.3%.) The *dihydrochloride* melted at 275–276°. (Found: C, 62.8; H, 8.3. $C_{24}H_{36}O_2N_2$,2HCl requires C, 63.0; H, 8.4%.)

1 : 8-*Bis*(p-*dimethylaminophenylmethylamino*)*octane* (No. 37). *p*-Dimethylaminobenzaldehyde and 1:8-diamino-octane treated in the usual manner gave 1:8-*bis*(p-*dimethylaminobenzylideneamino*)*octane* which crystallized from alcohol in yellow leaflets, m.p. 105–106°. (Found: C, 75.8; H, 9.3. $C_{26}H_{38}N_4$ requires C, 76.8; H, 9.4%.) Catalytic reduction furnished the *base*, long, fibrous needles from light petroleum, m.p. 65–66°. (Found: C, 75.7; H, 10.2; N, 13.2. $C_{26}H_{42}N_4$ requires C 76.1; H, 10.3; N, 13.65%.) The *tetrahydrochloride* formed a felted crystalline mass from alcohol, m.p. *ca.* 214° (decomp.). (Found: N, 9.3; Cl, 23.8. $C_{26}H_{42}N_4$, 4HCl requires N, 9.5; Cl, 23.9%.)

1:6-*Bis*(n-*heptylamino*)*hexane* (No. 38). *n*-Heptylamine (6.9 g.) was mixed with 1:6-dibromohexane (7.3 g.) and xylene (50 cc.). After standing overnight the mixture was heated under reflux for ½ hour. On cooling, the *dihydrobromide* separated; it forms plates from alcohol, m.p. 329°, yield 34%. (Found: C, 50.6; H, 9 8. $C_{20}H_{44}N_2$,2HBr requires C, 50.6; H, 9.8%.) The *dihydrochloride* crystallizes in shining plates from alcohol-acetone, m.p. 340°. (Found: C, 62.8; H, 11.95; N, 7.35; Cl, 18.5. $C_{20}H_{44}N_2$,2HCl requires C, 62.3; H, 12.0; N, 7.3; Cl, 18.4%.) The *dilactate* (No. 39) forms colourless needles from acetone, m.p. 105°. (Found: C, 63.0; H, 11.2. $C_{20}H_{44}N_2$,2$C_3H_6O_3$ requires C, 63.4; H, 11.45%.)

1:7-*Bis*(n-*heptylamino*)*heptane* (No. 40). The *dihydrobromide* was obtained in a similar manner from heptylamine and 1:7-dibromoheptane. It forms plates from alcohol, m.p. 318°. (Found: N, 5.8; Br, 32.8. $C_{21}H_{46}N_2$,2HBr requires N, 6.0; Br, 33.1%.) The *dilactate* crystallizes from alcohol-acetone, m.p. 143–146°. (Found: C, 64.5; H, 11.3. $C_{21}H_{26}N_2$,2$C_3H_6O_3$ requires C, 64.0; H, 11.2%.)

1:8-*Bis*(n-*heptylamino*)*octane* (No. 41). *n*-Heptylamine (4.8 g.) and 1:8-dibromo-octane (5.72 g.) were heated under reflux with acetone (30 cc.) until solid separated. After cooling, the *dihydrobromide* was collected. It forms leaflets from alcohol, m.p. 308°, yield 25%. (Found: N, 5.8; Br, 32.3. $C_{22}H_{48}N_2$,2HBr requires N, 5.45; Br, 31.8%.) The *dilactate* (No. 42) crystallizes from acetone in needles, m.p. 103–104°. (Found: C, 64.8; H, 11.5. $C_{22}H_{48}N_2$,2$C_3H_6O_3$ requires C, 64.6; H, 11.6%.)

1:9-*Bis*(n-*heptylamino*)*nonane* (No. 43). The *dihydrobromide* was obtained from *n*-heptylamine and 1:9-dibromononane in 25% yield by heating in xylene for 7 hours. It separates from alcohol in leaflets m.p. 305°. (Found: N, 5.6; Br, 31.4. $C_{23}H_{50}N_2$,2HBr requires N, 5.4; Br, 30.9%.) The *dilactate* forms needles from acetone, m.p. 132–134°. (Found: C, 65.15; H, 12.1. $C_{23}H_{50}N_2$,2$C_3H_6O_3$ requires C, 65.1; H, 11.7%.)

1:10-*Bis*(n-*heptylamino*)*decane* (No. 44). The *dihydrobromide* prepared similarly in 24% yield from *n*-heptylamine and 1:10-dibromodecane forms leaflets from alcohol, m.p. 320–322°. (Found: C, 54.55; H, 10.2; N, 5.5; Br, 30.2. $C_{24}H_{52}N_2$,2HBr requires C, 54.3; H, 10.3; N, 5.3; Br, 30.1%.) The *dilactate*, needles from dry alcohol-acetone, melts at 138–139°. (Found: C, 65.6; H, 11.8. $C_{24}H_{52}N_2$,2$C_3H_6O_3$ requires C, 65.7; H, 11.8%.)

1:10-*Bis*(n-*hexylamino*)*decane* (No. 45). The *dihydrobromide* was obtained in 45% yield from 1:10-dibromodecane and *n*-hexylamine (4 mols.) by heating for 16 hours in dry alcohol. It forms colourless needles, m.p. 315–317°. (Found: C, 53.0; H, 9.8; N, 6.0; Br, 31.9. $C_{22}H_{48}N_2$,2HBr requires C, 52.6; H, 10.0; N, 5.6; Br, 31.8%.) The *dilactate* separates from alcohol in feathery needles, m.p. 141–143°. (Found: C, 64.6; H, 11.6; N, 5.1. $C_{22}H_{48}N_2$,2$C_3H_6O_3$ requires C, 64.55; H, 11.6; N, 5.4%.)

1:8-*Bis*(n-*octylamino*)*octane* (No. 46). The *dihydrobromide*, obtained in 42% yield from 1:8-dibromo-octane and *n*-octylamine (4 mols.) in boiling alcohol (20 hours), forms plates, m.p. 314°. (Found: C, 54.2; H, 9.2; N, 5.5. $C_{24}H_{52}N_2$,2HBr requires C, 54.3; H, 10.3; N, 5.3%.) The *dilactate* (No. 47) separates in plates from alcohol, m.p. 105–106°. (Found: C, 65.4; H, 11.5; N, 4.7. $C_{24}H_{52}N_2$,2$C_3H_6O_3$ requires C, 65.7; H, 11.8; N, 5.1%.)

1 : 8-*Bis*(n-*nonylamino*)*octane* (No. 48). The *dihydrobromide* was obtained similarly from *n*-nonylamine and 1 : 8-dibromo-octane in platelets, m.p. 310–312°. (Found: C, 56.4; H, 9.3; N, 4.95; Br, 28.8. $C_{26}H_{56}N_2,2HBr$ requires C, 55.9; H, 10.5; N, 5.0; Br, 28.6%.) The *dilactate* (No. 48) forms short thin needles from dry alcohol-acetone, m.p. 109–111°. (Found: C, 66.4; H, 11.95; N, 4.5. $C_{26}H_{56}N_2,2C_3H_6O_3$ requires C, 66.6; H, 11.9; N, 4.9%.)

1 : 10-*Bis*(n-*undecylamino*)*decane* (No. 49). 1 : 10-Diaminodecane (4.3 g.) in amyl alcohol (26 cc.) was boiled under reflux and *n*-undecylbromide (11.8 g.) added gradually during 6 hours. Heating was continued for a further 18 hours. On cooling the *dihydrobromide* separated as a mass of crystals which after recrystallization from *iso*propylalcohol and from alcohol formed feathery needles, m.p. 302–305°, yield 20%. (Found: C, 59.85; H, 11.05; N, 4.6; Br, 25.2. $C_{32}H_{68}N_2,2HBr$ requires C, 59.7; H, 11.0; N, 4.4; Br, 24.9%.)

1 : 10-*Bis*(2′-*ethyl*-n-*hexylamino*)*decane* (No. 50). 1-Bromo-2-ethyl-*n*-hexane (19.3 g.) and 1 : 10-diaminodecane (8.6 g.) were heated under reflux in benzene (50 cc.) for 15 hours. After removing the benzene, the residue was heated for 15 minutes with 2% alcoholic sodium hydroxide, the alcohol distilled off and the bases extracted with ether. The residue from the ether was extracted with hot ligroin (b.p. 90–120°), cooled, filtered from diaminodecane and then treated with dry HCl. The *dihydrochloride* which separated formed fine needles from water, m.p. 128–132°, yield 10%. (Found: C, 66.3; H, 12.1; N, 6.0; Cl, 15.4. $C_{26}H_{56}N_2,2HCl$, requires C, 66.5; H, 12.4; N, 6.0; Cl, 15.1%.) The *dihydrobromide* crystallizes from alcohol in fine needles, m.p. 169–172°. (Found: C, 56.3; H, 10.5; N, 5.3; Br, 28.6. $C_{26}H_{56}N_2,2HBr$ requires C, 55.9; H, 10.5; N, 5.0; Br, 28.6%.)

1 : 10-*Bis*(*triallylcarbinamino*)*decane* (No. 51). 1 : 10-Dibromodecane was heated in alcoholic solution with 7 mols. of triallylcarbinamine (Henze, Allen and Leslie, 1943) for 24 hours. The solvent was removed and the crystalline residue shaken with ether and sodium hydroxide. The ether on evaporation left an oil from which excess of triallylcarbinamine was removed by distillation under reduced pressure at an oil bath temperature of 140°. The residue was converted to *dihydrochloride*, colourless crystals from water, m.p. 246–251° (decomp.). (Found: C, 70.0; H, 10.5; N, 5.5; Cl, 13.5. $C_{30}H_{52}N_2,2HCl$ requires C, 70.15; H, 10.6; N, 5.5; Cl, 13.8%.) The *dinitrate* forms clusters of crystals from water, m.p. 190° (decomp.). (Found: C, 63.8; H, 9.85; N, 10.0. $C_{30}H_{52}N_2,2HNO_3$ requires C, 63.6; H, 9.6; N, 9.9%.)

1 : 10-*Bis*(*geranylamino*)*decane* (No. 52). 1 : 10-Dibromodecane was heated under reflux for 24 hours with 4 parts by weight of geranylamine. Addition of ether precipitated a mixture of hydrobromides which on recrystallization from *iso*propylalcohol gave silky platelets of *bis*(*geranylamino*)*decane dihydrobromide*, m.p. 218°. (Found: C, 59.3; H, 9.4; N, 4.6; Br, 26.7. $C_{30}H_{56}N_2$, 2HBr requires C, 59.4; H, 9.6; N, 4.6; Br, 26.35%.)

1 : 9-*Bis*(n-*octylamino*)-2 : 8-*dimethylnonane* (No. 53). 2 : 8-Dimethyl-1 : 9-dibromononane (5.4 g.) and *n*-octylamine (17.78 g.) in dry alcohol (30 cc.) were heated under reflux for 16 hours, the solvent removed and the residue shaken with ether and alkali. After removing the ether, excess of octylamine was distilled off under reduced pressure (12.0 g. b.p. 96–98°/45 mm.) and the residue converted to *dihydrobromide*. After recrystallization from *iso*propyl alcohol the salt was obtained in 60% yield, m.p. 248–251° with previous sintering. (Found: C, 56.5; H, 10.35; N, 4.7. $C_{27}H_{58}N_2,2HBr$ requires C, 56.6; H, 10.6; N, 4.9%.)

1 : 10-*Bis*(n-*octylamino*)-2 : 9-*dimethyldecane* (No. 54). The *dihydrobromide* was obtained in a similar manner from 2 : 9-dimethyl-1 : 10-dibromodecane, in 50% yield. It forms fine needles from *iso*propyl alcohol, m.p. 242–244°, with previous sintering. (Found: C, 57.65; H, 10.7; N, 5.05. $C_{28}H_{60}N_2,2HBr$ requires C, 57.3; H, 10.7; N, 4.8%.)

1 : 8-*Bis*(cyclo*hexylamino*)*octane* (No. 55) was obtained from 1 : 8-dibromo-octane and *cyclo*hexylamine. After removal of excess *cyclo*hexylamine the *base* was distilled at about 200°/0.2 mm. (yield 84%). After a second distillation the base crystallized on cooling, m.p. 27–28°. (Found: N, 8.9. $C_{20}H_{40}N_2$ requires N, 9.1%.) The *dihydrochloride* forms colourless crystals from hot alcohol in which it is sparingly soluble, m.p. 284–285°. (Found: C, 63.0; H, 11.1; Cl, 18.6. $C_{20}H_{40}N_2,2HCl$ requires C, 63.0; H, 11.1; Cl, 18.6%.)

1 : 10-*Bis*(cyclo*hexylamino*)*decane* (No. 56) was obtained in a similar manner from 1 : 10-dibromodecane in 95% yield. The *base* boils at *ca.* 205°/0.2 mm. and crystallizes on cooling, m.p. 35–36.5°. (Found: C, 78.3; H, 13.1. $C_{22}H_{44}N_2$ requires C, 78.5; H, 13.2%.) The *dihydrochloride* forms colourless crystals from alcohol, m.p. 322° (decomp.). (Found: C, 64.7; H, 11.3; Cl, 17.45. $C_{22}H_{44}N_2,2HCl$ requires C, 64.5; H, 11.3; Cl, 17.3%.)

1 : 10-*N*: *N*′-*Dimorpholyldecane* (No. 57). 1 : 10-Dibromodecane (4.5 g.) was heated under reflux for 12 hours with morpholine (5.22 g.) and dry alcohol (15 cc.). Some morpholine hydrochloride separated. The mixture was made alkaline with NaOH and steam distilled to remove morpholine and alcohol. The residue was extracted with ether and the base converted to *dihydrochloride* which separates from dry alcohol in needles, m.p. 240–242° (decomp.) yield 84%. (Found: C, 56.15; H, 10.3; N, 7.4; Cl, 18.25. $C_{18}H_{36}O_2N_2,2HCl$ requires C, 56.1; H, 9.9; N, 7.3; Cl, 18.4%.)

1 : 10-*Bis*(β-*diethylaminoethylamino*)*decane* (No. 58). 1 : 10-Diaminodecane (8.2 g.) in benzene (50 cc.) was mixed with freshly prepared β-diethylaminoethyl chloride and left overnight. Potassium carbonate (7.9 g.) was added and the mixture heated under reflux for 20 hours. After removing the benzene the residue was shaken with ether and sodium hydroxide solution. The ether was dried and evaporated, and the residue distilled. A fraction b.p. 200–240°/6 mm. was collected and converted to *tetrahydrobromide*. It forms microscopic needles from amyl alcohol, m.p. 190–192° (3.5 g.). (Found: C, 37.9;

H, 8.0; N, 8.9; Br, 45.6. $C_{20}H_{50}N_4,4HBr$ requires C, 38.0; H, 7.8; N, 8.1; Br, 46.0%.)

1 : 5-*Bis*(n-*octylamino*)*pentane* (No. 59). Cadaverine (1.0 g.) in absolute alcohol (5 cc.) was heated under reflux for 7.5 hours with *n*-octyl bromide (3.8 g.). The *dihydrobromide* separated from alcohol in flat needles, m.p. 329–332° (decomp.), yield 15%. (Found: Br, 33.6. $C_{21}H_{46}N_2,2HBr$ requires Br, 32.7%.) By heating 1 : 5-dibromopentane with *n*-octylamine only *N*-octylpiperidine, b.p. 136–8/18 mm. was obtained. The hydrochloride had m.p. 189–191°. (c.f. v. Braun and Buchmann, 1931.)

1 : 3-*Bis*(n-*propylamino*)*propane* (No. 60). The *dihydrobromide* was obtained by adding 1 : 3-dibromopropane (20.2 g.) slowly during 2.5 hours to a boiling solution of *n*-propylamine (11.8 g.) in benzene (20 cc.). The mixture was heated for a further 4 hours, the hydrobromide filtered off and recrystallized from alcohol. It forms plates, m.p. 304° (7.0 g.). (Found: N, 8.75; Br, 49.7. $C_9H_{22}N_2,2HBr$ requires N, 8.75; Br, 50.0%.)

1 : 3-*Bis*(n-*heptylamino*)*propane* (No. 61). *n*-Heptylamine (11.5 g.) was mixed with 1 : 3-dibromopropane (10.1 g.) in benzene (10 cc.). The mixture developed heat; it was left to stand for several days, the mush of crystals filtered off and washed with water to remove heptylamine hydrochloride. The residue of 1 : 3-*bis*(n-*heptylamino*)*propane dihydrobromide* furnished platelets from alcohol, m.p. 320–322°. (Found: C, 47.25; H, 8.9; N, 6.75; Br, 37.25. $C_{17}H_{38}N_2,2HBr$ requires C, 47.2; H, 9.3; N, 6.5; Br, 37.0%.)

1 : 3-*Bis*(n-*tridecylamino*)*propane* (No. 62). The *dihydrobromide* was obtained in 65% yield by heating 1 : 3-dibromopropane and *n*-tridecylamine in dry alcohol for 18 hours. After two crystallizations from alcohol it formed platelets, m.p. 304–307°. (Found: N, 4.8. $C_{29}H_{62}N_2,2HBr$ requires N, 4.7%.) The *dilactate* separates from alcohol in rosettes of stout needles, m.p. 148–150°, very sparingly soluble in water. (Found: C, 68.0; H, 12.0; N, 4.8. $C_{29}H_{62}N_2,2C_3H_6O_3$ requires C, 67.9; H, 12.05; N, 4.5%.)

1 : 10-*Bis*(*di*-n-*butylamino*)*decane* (No. 64). This was obtained by the method of B.P. 433,086. The *acid succinate* separates from ethyl acetate in oily drops which slowly crystallize. After washing with acetone the salt had m.p. 81–86°. (Found: C, 64.5; H, 10.3. $C_{26}H_{56}N_2,2C_4H_6O_4$ requires C, 64.5; H, 10.8%.)

Summary

1. Several series of secondary diamines formally related to emetine have been prepared and tested against *Entamoeba histolytica* both *in vitro* and *in vivo*.

2. Selected members of the series have also been tested against experimental infections with trypanosomes, leishmania and malaria.

3. Bis (β - 3 : 4 - dimethoxyphenylethylamino) - alkanes in which the hydrocarbon chain contained 6 to 10 carbon atoms were active. The corresponding 4-monomethoxy compounds were less active.

4. Bis (β - phenylethylamino) alkanes also showed activity which was increased by the introduction of chlorine into the *ortho-* or *para-* positions of the benzene ring. The *ortho-* compound was most active but also more toxic. Bis(phenylalkylamino)alkanes with either a greater number or fewer carbon atoms between the nucleus and the amino group were less effective.

5. Bis(alkylamino)alkanes containing 7 or 8 carbon atoms in the alkyl groups and 6 to 10 carbon atoms in the connecting chain also showed activity. This was slightly higher *in vivo* than that of the bis(β-phenylethylamino)alkane series, but results at low dose-levels were erratic.

We are indebted to Dr. H. King for a gift of 1 : 10-bis(*n*-amylamino)decane dihydrochloride (No. 65). Our thanks are also due to Messrs. P. Amsden, P. Hankin, J. M. Judd, R. Nicholson, R. Penfound, F. J. Peters, K. Pratley, A. G. Turner, C. F. Varney and the Misses S. Pluthero and R. Shipman for much assistance in the experimental work.

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THE CHEMOTHERAPY OF AMOEBIASIS

PART III. VARIANTS OF BIS(DIAMYLAMINO)DECANE

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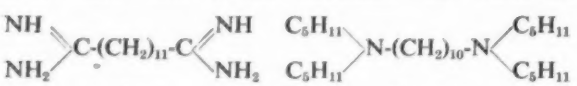
1:11-Diamidinoundecane (I) and 1:10-bis(di-*n*-amylamino)decane (II) are similarly constituted in that both contain two terminal basic radicals separated by a long chain of methylene groups. The former was shown by King, Lourie, and Yorke (1937) to be an active trypanocide in high dilution and the latter by Pyman (1937) to be an active amoebicide. Later Ashley, Barber, Ewins, Newbery, and Self (1942) modified the structure



I II

III

IV

V

of (I) by interrupting the chain with phenyl and phenylether groups, and obtained such compounds as 4:4′-diamidinostilbene ("stilbamidine") (III), 4:4′-diamidinodiphenoxy-propane ("propamidine") (IV) and -pentane ("pentamidine") (V) which were shown by Lourie and Yorke (1939) to have greatly enhanced trypanocidal activity.

We have now studied the effect on amoebicidal activity of similar changes in the structure of 1:10-bis(di-*n*-amylamino)decane, and of the corresponding secondary bases. A few derived quaternary ammonium salts have also been included. The methods of testing were those described in Part I by Goodwin, Hoare, and Sharp (1948), and the results are given in Table I.

Discussion of Results in Table I

The results with the standard substances 1:10-bis(di-*n*-butylamino)decane (No. 64) and 1:10-bis-(di-*n*-amylamino)decane (No. 65, II) showed the compounds to be much less active *in vitro* than Pyman's results indicated. This may be due to the fact that the cultures used by Pyman contained a mixed bactericidal flora, whereas our tests were made upon a culture of amoebae with a single strain of *Bact. coli*. These compounds had no significant activity *in vivo*.

The tertiary aromatic amines (Nos. 67–73) were found to be completely inactive, both *in vitro* at a concentration of 10^{-4} and *in vivo* in high doses, and a similar inactivity was found in the series of tertiary aromatic amines containing ether groupings (Nos. 74–81). These are weak bases, but the more strongly basic tertiary araliphatic compounds (Nos. 82–84) also showed no significant activity.

Since all the active compounds recorded in Part II were secondary amines a number of secondary aromatic amines were prepared (Nos. 85–92); here a slight *in vitro* activity became apparent when the alkyl group was amyl (No. 92) or heptyl (No. 89), but the compounds had practically no action *in vivo*. Nos. 86, 87, and 90, which, in addition to the two secondary amino-groups, carry two tertiary amino-groups, again had but slight activity. The length of the chain connecting the benzene rings seems to have little or no influence in these series.

In the group of secondary araliphatic amines (Nos. 93 to 106), the majority of the compounds had a moderate degree of *in vitro* activity, two showed a slight *in vivo* activity at high dose-levels (Nos. 103 and 104), and in two cases (Nos. 100 and 105) there was a high *in vitro* activity but no action *in vivo*. This group shows clearly that the *in vitro* test taken alone is inadequate as a means of assessing the value of a new drug. In the above instance activity is shown by a group of compounds therapeutically useless; in Part I it

TABLE I

Columns 6 and 7: "+" signifies improvement, "—" no improvement.
Columns 10 and 11: "—" signifies no activity at a concentration of 10^{-4}.

Column 12: "1" signifies no activity against *T. equiperdum*.
"2" " " " " *T. rhodesiense*.
"3" " " " " *T. congolense*.
"4" " " " " *T. cruzi*.
"5" " " " " *Leishmania donovani*.
"6" " " " " *Plasmodium gallinaceum*.

Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoebicidal conc.	Bact.-cidal conc.	
					Walls	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
$(C_4H_9)_2N(CH_2)_{10}N(C_4H_9)_2$, $2C_4H_6O_4$	64	500	930	0.5	—	—	3/6	50	10^{-4}	—	..
$(C_5H_{11})_2N(CH_2)_{10}N(C_5H_{11})_2$, 2HCl	65	..	..	0.5 0.2 0.1	+ + —	+ + —	3/7 7/14 3/12	40 50 25	10^{-5}	—	..
Tertiary aromatic amines											
$\left((C_4H_9)_2N\langle\bigcirc\rangle-\right)_2$, 2HCl	67	180	>2000	0.5 0.2 0.1	— — —	— — —	0/3 0/4 0/7	0 0 0	—	—	1,3,4, 5,6.
$\left((C_5H_{11})_2N\langle\bigcirc\rangle-\right)_2$, 2HCl	68	2000	2000	0.5	—	—	8/11	75	—	—	..
$\left((C_4H_9)_2N\langle\bigcirc\rangle-\right)_2CH_2$, 2HCl	69	950	>2000	0.5 0.2 0.1	Toxic — —	— —	2/8 0/7	25 0	—	—	1,4,6.
$\left((C_5H_{11})_2N\langle\bigcirc\rangle-\right)_2CH_2$, 2HCl	70	..	..	0.5	—	—	6/13	45	—	—	5.
$\left((C_4H_9)_2N\langle\bigcirc\rangle-\right)_2(CH_2)_2$, 2HCl	71	350	>2000	0.1	—	—	3/10	30	—	—	1,6.
$\left(C_5H_{11})_2N\langle\bigcirc\rangle-\right)_2(CH_2)_2$, 2HCl	72	>2000	>2000	0.5	—	—	1/5	20	—	—	1,3.
$\left((C_4H_9)_2N\langle\bigcirc\rangle CH=\right)_2$,2HCl	73	350	>2000	0.2	—	—	0/6	0	—	—	1,5,6.
Tertiary amino phenyl ethers											
$\left((C_4H_9)_2N\langle\bigcirc\rangle-\right)_2O$, 2HCl	74	230	>2000	0.5 0.2	— —	— —	0/6 1/5	0 20	—	—	..
$\left((C_5H_{11})_2N\langle\bigcirc\rangle-\right)_2O$, 2HCl	75	165	>2000	0.5	—	—	0/6	0	—	—	1,3.
$\left((C_4H_9)_2N\langle\bigcirc\rangle O-\right)_2CH_2$, 2HCl	76	2000	>2000	0.5	—	—	0/4	0	—	—	1,3.

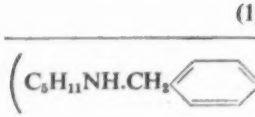
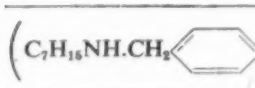
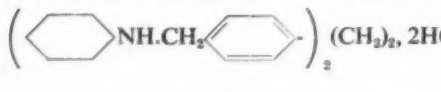
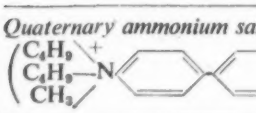
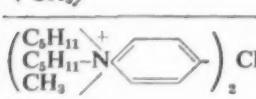
TABLE I—*continued*

Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoebicidal conc.	Bact.-cidal conc.	
					Walls	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
$\left((C_5H_{11})_2N\text{-}C_6H_3\text{-}O\text{-}\right)_2CH_2$, 2HCl	77	470	350	0.5 0.2	— —	— —	4/7 3/5	55 60	—	—	..
$\left((C_4H_9)_2N\text{-}C_6H_3\text{-}O\text{-}\right)_2(CH_2)_2$, 2HCl	78	600	>2000	0.2 0.1	— —	— —	2/8 0/8	25 0	—	—	4,5.
$\left((C_5H_{11})_2N\text{-}C_6H_3\text{-}O\text{-}\right)_2(CH_2)_2$, 2HCl	79	800	>2000	0.5 0.2 0.1	— + —	— + —	1/3 0/4 3/7	35 0 40	—	—	1,3, 5,6.
$\left((C_4H_9)_2N\text{-}C_6H_3\text{-}O\text{-}\right)_2(CH_2)_3$, 2HCl	80	2000	>2000	0.5	—	—	2/4	50	—	—	..
$\left((C_5H_{11})_2N\text{-}C_6H_3\text{-}O\text{-}\right)_2(CH_2)_3$, 2HCl	81	950	>2000	0.5	—	—	2/8	25	—	—	..
$\left((C_2H_5)_2N(CH_2)_2O\text{-}C_6H_4\text{-}\right)_2$, 2HCl	82	70	270	0.2	—	—	0/6	0	—	—	1,3,5.
Tertiary araliphatic amines											
$\left((C_4H_9)_2N.CH_2\text{-}C_6H_4\text{-}\right)_2$, 2HBr	83	350	>1000	0.5 0.2	Toxic —	— —	1/8	10	—	—	4,6.
$\left(C_5H_{10}N.CH_2\text{-}C_6H_4\text{-}\right)_2CH_2$, 2HCl	84	..	..	0.5 0.2	+ —	+ —	Toxic 3/3 0/8	100 0	10^{-4}	—	..
Secondary aromatic amines											
$\left(C_6H_5NH\text{-}C_6H_4\text{-}\right)_2$, 2HCl	85	250	>2000	0.2	—	—	0/6	0	—	—	1,5,6.
$\left((C_2H_5)_2N(CH_2)_2NH\text{-}C_6H_4\text{-}\right)_2$, 3HCl	86	570	330	0.5 0.2	— —	— —	1/5 1/5	20 20	10^{-4}	—	2,3.
$\left((C_2H_5)_2N(CH_2)_3NH\text{-}C_6H_4\text{-}\right)_2$, 4HBr	87	..	..	0.5 0.2	+ —	+ —	3/8 2/7	40 30	10^{-4}	—	(6).
$\left(C_6H_5NH\text{-}C_6H_4\text{-}\right)_2CH_2$, 2HCl	88	300	>2000	0.2 0.1	— —	— —	0/3 1/9	0 10	—	—	1,6.
$\left(C_7H_{15}NH\text{-}C_6H_4\text{-}\right)_2CH_2$, 2HCl	89	>2000	>2000	0.5 0.2 0.1	+ — —	+ — —	Toxic 2/8 0/6	 25 0	—	—	..

TABLE I—*continued*

Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoe-bicidal conc.	Bact.-cidal conc.	
					Walls	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
$\left((C_2H_5)_2N(CH_2)_2NH\text{-}C_6H_4\right)_2CH_2$, 4HCl	90	..	1400	0.5	—	—	0/6	0	10^{-4}	—	5,6.
$\left(C_4H_9NH\text{-}C_6H_4\right)_2(CH_2)_2$, 2HCl	91	..	..	..	..	..	..	..	—	—	6.
$\left(C_5H_{11}NH\text{-}C_6H_4\right)_2(CH_2)_2$, 2HCl	92	>2000	>2000	0.5 0.2	+ —	+ —	1/4 4/5	25 80	10^{-4}	—	1,3,6.
Secondary araliphatic amines											
$C_3H_7NH.CH_2C_6H_4CH_2NH.C_3H_7$, 2HCl	93	550	450	0.15	—	—	0/6	0	—	—	6.
$C_4H_9NH.CH_2C_6H_4CH_2NH.C_4H_9$, 2HCl	94	350	310	0.5 0.2	— —	— —	2/6 1/5	35 20	—	—	2,3,6.
$C_5H_{11}NH.CH_2C_6H_4CH_2NH.C_5H_{11}$, 2HCl	95	230	175	0.5	—	—	0/4	0	—	—	2,3.
$\left(C_4H_9NH.CH_2C_6H_4\right)_2$, 2HCl	96	1500	1500	0.2	—	—	1/3	35	10^{-4}	—	2,3,6.
$\left(C_6H_5NH.CH_2C_6H_4\right)_2$, 2HCl	97	930	810	0.5 0.2 0.1	— — —	— — —	Toxic 2/5 3/7	 40 40	10^{-4}	—	..
$\left(C_4H_9NH.CH_2C_6H_4\right)_2CH_2$, 2HCl	98	..	..	0.5 0.2	— —	— —	0/3 2/4	0 50	10^{-4}	—	1,3.
$\left(C_5H_{11}NH.CH_2C_6H_4\right)_2CH_2$, 2HCl	99	933	574	0.2	—	—	0/4	0	10^{-4}	—	6.
$\left(C_6H_5NH.CH_2C_6H_4\right)_2CH_2$, 2HCl	100	..	..	0.5	—	—	2/4	50	—	—	5,6.
$\left(C_6H_5CH_2NH.CH_2C_6H_4\right)_2CH_2$,2HCl	101	>2000	>2000	0.5	—	—	0/4	0	10^{-4}	—	..
$\left(C_3H_7NH.CH_2C_6H_4\right)_2(CH_2)_2$, 2HCl	102	>2000	270	0.2	—	—	2/13	15	—	—	2,3.
$\left(C_4H_9NH.CH_2C_6H_4\right)_2(CH_2)_2$, 2HCl	103	80	120	0.5 0.2	— —	— —	5/8 0/4	60 0	10^{-4}	—	..

TABLE I—*continued*

Substance	Ref. No.	Approx. LD50 (mg./kg.)		Amoebicidal tests							Other tests
				in vivo					*in vitro*		
		Oral	Sub-cut.	% diet	Caecal condition		Rats clear	%	Amoebicidal conc.	Bact.-cidal conc.	
					Walls	Conts.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	104	..	250	0.5 0.2	+ –	+ –	3/4 1/8	75 10	10^{-5}	–	..
	105	>2000	>2000	0.2	–	–	0/1	Toxic	10^{-5}	–	6.
	106	470	1000	0.5 0.2 0.1	Toxic + +	 + +	 2/5 4/7	 40 55	10^{-5}	–	..
Quaternary ammonium salts 	107	>2000	550	0.5 0.2	Toxic –	 –	 4/5	 80	10^{-4}	–	3.
	108	440	100	0.2 0.1 0.05	+ – –	– – –	6/6 0/4 0/2	100 0 0	10^{-4}	–	2,3.
,, $(CH_2)_2.\ I_2^-$	109	20	..	0.5	–	–	1/10	10	10^{-4}	–	1,3,6.
	110	..	..	0.5	–	–	4/8	50	..	..	..

was shown that diodoquin, although active *in vivo*, had no appreciable *in vitro* activity.

The quarternary ammonium salts (Nos. 107–110) were slightly more active than the corresponding tertiary bases (Nos. 67, 70, 72, and 84).

Chemical Section

Aromatic amines

NNN'N'-Tetra-n-*butylbenzidine* (No. 67). Benzidine (1.84 g., anhydrous) was refluxed in *n*-butanol with a 33% excess of *n*-butylbromide (7.3 g.) and potassium carbonate (3.7 g.) for 16 hours. The filtered solution was evaporated and the residue shaken with ether and aqueous sodium hydroxide to isolate the crude product, which was then dissolved in light petroleum (b.p. 40–60°) and passed through a column of activated alumina. The *base* (3.2 g., 78%), which exhibited a blue fluorescence in organic solvents, was obtained on evaporation of the petrol solution; it crystallized from alcohol in flakes m.p. 58.5°. (Found: C, 82.2; H, 10.8. $C_{28}H_{44}N_2$ requires C, 82.3; H, 10.9%). The *dipicrate*, which crystallized in yellow flakes from alcohol, had m.p. 203–204° (decomp.). (Found: C, 55.4; H, 5.9; N, 12.9. $C_{28}H_{44}N_2, 2C_6H_3O_7N_3$ requires C, 55.4; H, 5.8; N, 12.9%). The *dihydrochloride*, m.p. 240–250° (decomp.), was readily soluble in alcohol but suffered partial hydrolysis in aqueous solution. (Found: N, 6.1; Cl, 14.9. $C_{28}H_{44}N_2$, 2HCl requires N, 5.8; Cl, 14.8%). The base reacted with methyl iodide in benzene to form a *monomethiodide* (No. 107) which crystallized from ethanol, m.p. 146–147° (after drying at 100° *in vacuo*). (Found: C, 63.3; H, 8.5; I, 24.3. $C_{28}H_{44}N_2,CH_3I$ requires C, 63.3; H, 8.6; I, 23.1%).

An alternative method of obtaining tetra-*n*-butylbenzidine utilized the oxidative procedure of Ullmann and Dieterle (1904) and of Fröhlich (1911): Di-*n*-butylaniline (4.1 g.), concentrated sulphuric acid (20 g.), and turpentine (0.1 g.) were heated together at 190–200° for 4½ hours. The mixture was made alkaline and, after removal of unchanged dibutylaniline by steam-distillation, the residual mass was dried, powdered and extracted with

benzene (Soxhlet). The dark extract was evaporated and the residue, decolorized by filtration of its solution in light petroleum through alumina, consisted of NNN′N′-tetra-*n*-butylbenzidine (2.6 g., 64%).

NN′-Di-n-*butylbenzidine* (No. 85). When benzidine was butylated with only half the above quantity of *n*-butyl bromide the resulting mixture of bases was only partially soluble in light petroleum (b.p. 40–60°). When the undissolved substance was dissolved in benzene and passed through a column of alumina some unchanged benzidine remained in the column, while the benzene carried through *NN′-di*-n-*butylbenzidine*, which crystallized from alcohol in laminae m.p. 72°. (Found: C, 81.0; H, 9.8. $C_{20}H_{28}N_2$ requires C, 81.0; H, 9.5%.) The *dihydrochloride* crystallized from ethanol in fine needles m.p. *ca.* 280° (decomp.). (Found: C, 64.4; H, 8.4. $C_{20}H_{28}N_2$,2HCl requires C, 65.0; H, 8.2%.)

The base was also obtained in small yield by the oxidation of *n*-butylaniline with sulphuric acid in the manner previously described.

NNN′N′-Tetra-n-*amylbenzidine* (No. 68). The amylation of benzidine was carried out in a similar manner to the butylation, using a 33% excess of *n*-amylbromide in *n*-butanol. The *base* obtained on removal of the petroleum was distilled at *ca.* 270°/0.5 mm., and was obtained as an oil. (Found: C, 83.0; H, 11.5; N, 6.3. $C_{32}H_{52}N_2$ requires C, 82.7; H, 11.3; N, 6.0%.) The *dipicrate* crystallized from ethanol in small yellow prisms m.p. 186–187°. (Found: C, 57.4; H, 6.3; N, 12.5. $C_{32}H_{52}N_2$, $2C_6H_3O_7N_3$ requires C, 57.3; H, 6.3; N, 12.1%.) The *dihydrochloride*, m.p. 230–235° (decomp.), crystallized from ethanol on addition of ether. (Found: C, 70.9; H, 10.0. $C_{32}H_{52}N_2$,2HCl requires C, 71.5; H, 10.1%.)

Bis(p-*NN-di*-n-*butylaminophenyl*)*methane* (No. 69) This was prepared according to Reid and Lynch (1936) and, in 75% yield, by the butylation of bis(*p*-aminophenyl)methane. The base was obtained as an oil, b.p. 280–320°/20 mm. (Found: C, 82.7; H, 11.4. Calc.: C, 82.4; H, 11.0%.) The dipicrate, golden flakes from ethanol, had m.p. 183–184° (Reid and Lynch, 1936, recorded m.p. 156°). (Found: C, 55.8; H, 5.9; N, 12.8. Calc.: C, 55.9; H, 6.0; N, 12.7%.) The *dihydrochloride* m.p. *ca.* 220° (decomp.) was very soluble in ethanol and in water. (Found: Cl, 14.6. $C_{29}H_{46}N_2$,2HCl requires Cl, 14.3%.)

Bis(p-*NN-di*-n-*amylaminophenyl*)*methane* (No. 70). The *base*, obtained by amylation of bis(*p*-aminophenyl)-methane with a 33% excess of *n*-amylbromide in the manner previously described, formed a viscous oil. (Found: C, 83.1; H, 11.2. $C_{33}H_{54}N_2$ requires C, 82.8; H, 11.4%.) The *dihydrochloride* crystallized from ethanol, m.p. 203° (decomp.). (Found: Cl, 13.0. $C_{33}H_{54}N_2$,2HCl requires Cl, 12.9%.) The *dimethiodide*, flat jagged needles from ethanol, had m.p. *ca.* 140° in a sealed tube (softening at 70–75°). (Found: N, 3.8; I, 33.4. $C_{33}H_{54}N_2$,2CH_3I requires N, 3.7; I, 33.3%.) The dimethochloride (No. 108) was prepared from this by the usual procedure.

Butylation of α: β-*bis*(p-*aminophenyl*)*ethane*. Butylation carried out by the standard method previously described gave an oil which distilled at *ca.* 230°/0.01 mm. The distillate, α: β-*bis*(p-*NN-di*-n-*butylaminophenyl*)*ethane* (No. 71), crystallized from ethanol in laminae m.p. 34.5–35.5°. (Found: C, 82.5; H, 11.1; N, 6.7. $C_{30}H_{48}N_2$ requires C, 82.5; H, 11.1; N, 6.4%.) The base and its solutions in ether or alcohol exhibited a strong violet fluorescence. The *dipicrate* crystallized from ethanol in golden needles or laminae m.p. 173.5–174.5° (slow heating); with rapid heating dimorphic change occurred at 174–175° and the m.p. was 188–189°. (Found: C, 56.7; H, 6.3; N, 12.6. $C_{30}H_{48}N_2$,2$C_6H_3O_7N_3$ requires C, 56.4; H, 6.1; N, 12.5%.) The *dihydrochloride*, soluble in ethanol and in water, had m.p. *ca.* 231° (decomp.). (Found: Cl, 13.9. $C_{30}H_{48}N_2$,2HCl requires Cl, 13.1%.)

When α: β-bis(*p*-aminophenyl)ethane (11.5 g.) was heated with *n*-butylbromide (35 g., 16% excess) in an autoclave at 200° for 40 hours the resulting mixture of bases reacted readily with acetic anhydride to give an oily product which was treated several times with warm light petroleum (b.p. 40–60°). Solid material crystallized from the petrol on standing and, on evaporation, the filtered solution gave an oil which was mainly α: β-bis(*p*-NN-di-*n*-butylaminophenyl)ethane. The solid material (12.8 g.) was crystallized from benzene/light petroleum to give α: β-*bis*(p-*N*-n-*butylacetamidophenyl*)-*ethane* in needles m.p. 85–86°. (Found: C, 76.2; H, 9.0; N, 6.8. $C_{26}H_{36}O_2N_2$ requires C, 76.4; H, 8.9; N, 6.9%.) On deacetylation with alcoholic hydrochloric acid this derivative gave α: β-*bis*(p-*N*-n-*butylaminopheryl*)*ethane* (No. 91) which formed crystals m.p. 86–87° from aqueous ethanol. (Found: C, 81.3; H, 9.6; N, 8.6. $C_{22}H_{32}N_2$ requires C, 81.5; H, 9.9; N, 8.6%.) The dihydrochloride had m.p. *ca.* 235° (decomp.). (Found: Cl, 18.3. $C_{22}H_{32}N_2$,2HCl requires Cl, 17.8%.)

Amylation of α: β-*bis*(p-*aminophenyl*)*ethane*. The standard amylation procedure did not in this case bring about complete reaction; a further treatment with *n*-amyl bromide was found to be necessary. α: β-*Bis*(p-*NN-di*-n-*amylaminophenyl*)*ethane* (No. 72) crystallized from 80% alcohol in jagged needles m.p. 40° showing a strong violet fluorescence. (Found: C, 83.0; H, 11.6. $C_{34}H_{56}N_2$ requires C, 83.0; H, 11.5%.) The *dipicrate*, yellow needles from ethanol, had m.p. 207–208°. (Found: C, 58.0; H, 6.7; N, 12.1. $C_{34}H_{56}N_2$,2$C_6H_3O_7N_3$ requires C, 58.1; H, 6.6; N, 11.8%), and was used in separating the pure base from the incompletely amylated reaction product. The *dihydrochloride* had m.p. 214–215°. (Found: Cl, 12.4. $C_{34}H_{56}N_2$,2HCl requires Cl, 12.5%.) The *dimethiodide* (No. 109) separated as a crystalline powder m.p. 158–160° (decomp.) on addition of benzene to its alcoholic solution. (Found: C, 55.2; H, 8.0; I, 32.3. $C_{34}H_{56}N_2$,2CH_3I requires C, 55.7; H, 8.1; I, 32.7%.)

When the products of the incomplete reaction in light petroleum (b.p. 40–60°) were passed through a column of alumina, α: β-*bis*(p-*N*-n-*amylaminophenyl*)*ethane* (No. 92) was adsorbed. The base was recovered by elution with ether/light petroleum and crystallized from ethanol in laminae m.p. 86°. (Found: C, 81.8; H, 10.2; N, 8.2. $C_{24}H_{36}N_2$ requires C, 81.8; H, 10.3; N, 8.0%.) The *dihydrochloride* had m.p. *ca.* 240° (decomp.). (Found:

N, 7.0; Cl, 17.0. $C_{24}H_{36}N_2$,2HCl requires N, 6.6; Cl, 16.7%.)

Trans-4:4′-*bis*(*di*-n-*butylamino*)*stilbene* (No. 73). This was obtained on applying the standard butylation procedure to *trans*-4:4′-diaminostilbene. The *base*, which exhibited a strong violet fluorescence in organic solvents, crystallized from ethanol in opaque laminae m.p. 70–71°. (Found: C, 82.8; H, 10.7; N, 6.6. $C_{30}H_{46}N_2$ requires C, 82.9; H, 10.7; N, 6.5%.) The *dipicrate*, small golden needles from much ethanol, had m.p. *ca.* 213° (decomp.). (Found: C, 56.6; H, 6.0; N, 12.4. $C_{30}H_{46}N_2$,2$C_6H_3O_7N_3$ requires C, 56.5; H, 5.9; N, 12.6%.) The *dihydrochloride* had m.p. 240–242°. (Found: N, 5.7: Cl, 13.9. $C_{30}H_{46}N_2$,2HCl requires N, 5.5; Cl, 14.0%.)

Tertiary aromatic amines derived from phenyl ethers

Bis(p-*aminophenoxy*)*alkanes*. The bis(*p*-aminophenoxy)alkanes used were prepared by the method described by Kinzel (1898) for the preparation of α:β-bis(*p*-aminophenoxy)ethane. αγ-*Bis*(p-*nitrophenoxy*)*propane* from sodium *p*-nitrophenate and 1:3-dibromopropane forms anhydrous crystals from alcohol, m.p. 132°. (Found: C, 56.6; H, 4.8; N. 8.8. $C_{15}H_{14}O_6N_2$ requires C, 56.6; H, 4.4; N, 8.8%.) αγ-Bis(*p*-acetamidophenyl)-propane, m.p. 183°, was obtained in 87% yield by reduction of the above nitro compound with powdered iron and acetic acid (99%). On hydrolysis with hydrochloric acid it yielded αγ-*bis*(p-*aminophenyl*)*propane dihydrochloride*, m.p. 268° after recrystallization from water, yield 75%. (Found: C, 54.6; H, 6.2; N, 8.2; Cl, 21.4. $C_{15}H_{18}O_2N_2$,2HCl requires C, 54.4; H, 6.1; N, 8.5; Cl, 21.4%.)

Bis(*dialkylaminophenyl*)*ethers* (Nos. 74–81). The general method for the preparation of these compounds is illustrated by the following example. To a boiling solution of 4:4′-diaminodiphenylether (4 g.) and *n*-butylbromide (22 g.) in alcohol (25 cc.), a solution of sodium (2.5 g.) in alcohol (80 cc.) was added at such a rate as to maintain a slightly alkaline reaction. After 12 hours the alcohol was removed and the residue diluted with water and extracted with ether. After distilling off the solvent the base was dissolved in light petroleum (b.p. 40–60°), passed through a column of alumina and purified as picrate. The picrate was then converted to hydrochloride in the usual manner. The properties of the compounds are given in Table II.

4:4′-*Bis*(β-*diethylaminoethoxy*)*diphenyl* (No. 82). 4:4′-Dihydroxydiphenyl (6.2 g.) was treated with sodium (1.6 g.) in ethanolic solution. The solution was refluxed overnight with β-diethylaminoethyl chloride (from 14.3 g. of the hydrochloride). The concentrated solution was made alkaline and extracted with ether; the resulting oil (3.5 g.). dissolved in light petroleum (b.p. 40–60°) was passed through a column of alumina. The *base* (2.9 g., 23%) obtained on removal of the solvent gave crystals, m.p. 45.5°, from aqueous alcohol. (Found: C, 75.0; H, 9.4; N, 7.4. $C_{24}H_{36}O_2N_2$ requires C, 75.0; H, 9.4; N, 7.3%.) The *dihydrochloride* had m.p. 244–245° (decomp.). (Found: C, 63.4; H, 8.6; Cl, 15.7. $C_{24}H_{36}O_2N_2$,2HCl requires C, 63.0; H, 8.4; Cl, 15.5%.)

Tertiary araliphatic amines

4:4′-*Bis*(*di*-n-*butylaminomethyl*)*diphenyl* (No. 83). 4:4′-Bis(bromomethyl)diphenyl (3.4 g.) and dibutylamine (2.75 g.) in toluene (50 cc.) were heated under reflux for 10 hours. The somewhat oily solid was filtered off, washed with hot toluene and recrystallized from acetone. The *dihydrobromide* separated in long shining rods, m.p. 227°. (Found: C, 60.45; H, 9.0; Br, 26.3. $C_{30}H_{48}N_2$,2HBr requires C, 60.2; H, 8.4; Br, 26.7%.)

4:4′-*Bis*(*N-piperidinomethyl*)*diphenylmethane* (No. 84). A solution of 4:4′-bis(bromomethyl)diphenylmethane (7.1 g.) and piperidine (3.4 g.) in ethanol (35 cc.) was refluxed for 7 hours, concentrated, and shaken with 10% sodium hydroxide and benzene. The benzene solution

TABLE II

Compound	No.	m.p.	Found %				Formula	Requires		
			C	H	N	Cl		C	H	N
Bis(p-*di*-n-*butylaminophenyl*)*ether*,2*HCl*	74		65.5	9.3		13.9	$C_{28}H_{44}ON_2$,2HCl, H_2O	65.2	9.4	
do. *dipicrate*		182°	54.4	5.7	13.3		$C_{28}H_{44}ON_2$,2$C_6H_3O_7N_3$	54.4	5.7	12
Bis(p-*di*-n-*amylaminophenyl*)*ether dipicrate*	75	148°	56.2	6.2	12.3		$C_{32}H_{52}ON_2$,2$C_6H_3O_7N_3$	56.3	6.2	11
Bis(p-*di*-n-*butylaminophenoxy*)*methane* 2*HCl*	76	88°[1]	62.3	9.4	5.15	12.7	$C_{29}H_{46}O_2N_2$,2HCl,2H_2O	62.0	9.0	5
do. *dipicrate*		168°	53.95	5.65	12.4		$C_{29}H_{46}O_2N_2$,2$C_6H_3O_7N_3$	53.95	5.7	12
Bis(p-*di*-n-*amylaminopheroxy*)*methane* 2*HCl*	77	84°[2]	63.95	9.5	4.7	11.5	$C_{33}H_{54}O_2N_2$,2HCl,2H_2O	63.95	9.8	4
do. *dipicrate*		140°	55.5	6.2	12.1		$C_{33}H_{54}O_2N_2$,2$C_6H_3O_7N_3$	55.9	6.2	11
αβ-*Bis*(p-*di*-n-*butylaminophenoxy*)*ethane* 2*HCl*	78	205°[3]			5.2	13.1	$C_{30}H_{48}O_2N_2$, 2HCl			5
do. *dipicrate*		156°	54.4	5.9	11.9		$C_{30}H_{48}O_2N_2$,2$C_6H_3O_7N_3$	54.4	5.9	12
αβ-*Bis*(p-*di*-n-*amylaminophenoxy*)*ethane* 2*HCl*	79	181°	68.0	9.65	5.0	11.4	$C_{34}H_{56}O_2N_2$, 2HCl	68.3	9.8	4
do. *dipicrate*		170°	56.3	6.7	11.6		$C_{34}H_{56}O_2N_2$,2$C_6H_3O_7N_3$	56.2	6.4	11
αγ-*Bis*(p-*di*-n-*butylaminophenoxy*)*propane* 2*HCl*	80	169°[4]	66.8	9.3	5.0	12.6	$C_{31}H_{50}O_2N_2$,2HCl	67.0	9.4	5
do. *dipicrate*		202°	54.9	5.9	12.3		$C_{31}H_{50}O_2N_2$,2$C_6H_3O_7N_3$	54.9	6.0	11
αγ-*Bis*(p-*di*-n-*amylaminophenoxy*)*propane* 2*HCl*	81	94°			4.8	11.5	$C_{35}H_{58}O_2N_2$,2HCl			4
do. *dipicrate*		198°	56.95	6.7	11.25		$C_{35}H_{58}O_2N_2$,2$C_6H_3O_7N_3$	56.6	6.5	11

[1]The anhydrous salt melts at 130°. [2]The anhydrous salt melts at 180°. [3]The dihydrate melts at 112°. [4]The dihydrate melts

gave an oil (3.1 g.) which was dissolved in light petroleum (b.p. 40–60°) and filtered through alumina. The resulting *base*, after distillation at 230°/0.5 mm., formed crystals m.p. 63–65° from aqueous ethanol. (Found: C, 82.0; H, 8.9; N, 7.5. $C_{25}H_{34}N_2$ requires C, 82.8; H, 9.5; N, 7.7%.) The *dipicrate*, orange-yellow needles from much ethanol, had m.p. 181–182° (after drying at 100° *in vacuo*). (Found: N, 13.8. $C_{25}H_{34}N_2,2C_6H_3O_7N_3$ requires N, 13.7%.) The *dihydrochloride* crystallized from alcohol-ether, m.p. *ca.* 300° (decomp.). (Found: C, 68.8; H, 7.8; Cl, 16.0. $C_{25}H_{34}N_2,2HCl$ requires C, 68.9; H, 8.3; Cl, 16.3%.) The *dimethiodide* separated from alcohol-benzene in yellowish crystals, m.p. 175° (decomp.). (Found: C, 50.6; H, 6.2. $C_{25}H_{34}N_2,2CH_3I$ requires C, 50.2; H, 6.2%.)

Secondary aromatic amines

NN′-Bis(β-*diethylaminoethyl*)*benzidine* (No. 86). Benzidine (4.4 g.) was refluxed overnight with β-diethylaminoethyl chloride (from 12 g. of the hydrochloride) and potassium carbonate (5 g.) in xylene. The solvent was removed by steam-distillation and a basic oil (11 g.) recovered by extraction with ether from alkaline solution. Partial purification was effected by passing the oil dissolved in light petroleum (b.p. 40–60°) through a column of alumina. The product (9.4 g.) was treated in ethanol with picric acid (11.5 g.) and the oily picrate which separated was extracted several times with acetone/ethanol. The extracts, on evaporation and basification, gave an oil from which an ether-insoluble hydrochloride (3.8 g.) was obtained. The *base* was obtained from this by treatment with ammonia; it crystallized in soft flakes m.p. 70° from aqueous ethanol. (Found: C, 75.3; H, 9.7; N, 14.6. $C_{24}H_{38}N_4$ requires C, 75.3; H, 10.0; N, 14.7%.) The *trihydrochloride*, soluble in ethanol, had m.p. *ca.* 235°. (Found: N, 21.8; Cl, 11.5. $C_{24}H_{38}N_4,3HCl$ requires N, 21.6; Cl, 11.4%.)

NN′-Bis(γ-*diethylaminopropyl*)*benzidine* (No. 87). Benzidine (4.5 g.) was refluxed with γ-diethylaminopropyl chloride and potassium carbonate in *n*-butanol, the method of procedure being that of the previous experiment. The oil (6.0 g.) obtained from the petrol solution gave a picrate (3.4 g.) when treated with picric acid (4.3 g.) in ethanol. The *base* (1.5 g.) obtained from the picrate was distilled at *ca.* 320°/0.4 mm. (Found: C, 76.4; H, 11.4; N, 13.3. $C_{26}H_{42}N_4$ requires C, 76.1; H, 10.3; N, 13.6%.) It formed a tetrahydrobromide as reported by Work (1940) and a *tripicrate* which separated as golden-yellow crystals m.p. 125° (decomp.) from acetone. (Found: C, 46.4; H, 4.8; N, 15.8. $C_{26}H_{42}N_4$, $3C_6H_3O_7N_3,2H_2O$ requires C, 46.6; H, 4.7; N, 16.1%.)

Bis(p-*N*-n-*butylaminophenyl*)*methane* (No. 88). This compound was obtained from *n*-butylaniline and formaldehyde as a crystalline solid m.p. 44–45°. (Wagner, 1934, gave m.p. 41–42°; Reid and Lynch, 1936, recorded m.p. 45°.) The *dihydrochloride* had m.p. 160–162°. (Found: C, 65.4; H, 8.5. $C_{21}H_{30}N_2,2HCl$ requires C, 65.7; H, 8.4%.)

Bis(p-*N*-n-*heptylaminophenyl*)*methane* (No. 89). Bis(*p*-aminophenyl)methane (2.47 g.) was boiled with sodamide (1 g.) in dry xylene for 4 hours; *n*-heptylbromide (4.5 g.) was added, and the mixture refluxed overnight. The solvent was removed by steam-distillation and the reaction product extracted with ether from alkaline solution. The resulting dark-coloured oil was dissolved in light petroleum (b.p. 40–60°) and the solution passed twice through a column of alumina. The colourless oil (4.0 g.) obtained on removal of solvent was distilled at 0.2 mm., collecting material distilling up to 280°. This crude base was treated in ethanol with a little 10% hydrochloric acid, causing the separation of an oily hydrochloride which on standing at 0° formed a semi-solid mass. This was pressed on a porous tile and crystallized from ethanol/ether to give bis(p-*N*-n-*heptylaminophenyl*)*methane dihydrochloride* (0.9 g., 15%) m.p. 195–196°. (Found: C, 69.1; H, 9.5. $C_{27}H_{42}N_2$, 2HCl requires C, 69.4; H, 9.5%), which readily became blue in moist air. The *base* was purified by alumina (in light petroleum/benzene) and formed crystals m.p. 48° from aqueous ethanol. (Found: C, 81.7; H, 10.7; N, 6.9. $C_{27}H_{42}N_2$ requires C, 82.2; H, 10.7; N, 7.1%.)

Bis(p-β-*diethylaminoethylaminophenyl*)*methane* (No. 90). Bis(*p*-aminophenyl)methane (9.9 g.) was refluxed overnight with β-diethylaminoethyl chloride (from 18.1 g. of the hydrochloride) and potassium carbonate (7.2 g.) in ethanol. The resulting basic oil (17 g.), purified by alumina (light petroleum solution) was distilled at 0.2 mm., collecting material (7.3 g.) distilling above 240°. This product was purified by recrystallization of the tetrahydrochloride, m.p. *ca.* 167–169° from ethanol. The salt was too deliquescent for satisfactory analyses to be obtained. The *base* was an oil b.p. *ca.* 280°/0.2 mm. (Found: C, 75.8; H, 10.2; N, 13.7. $C_{25}H_{40}N_4$ requires C, 75.7; H, 10.2; N, 14.1%.)

Secondary araliphatic amines

Preparation of aromatic bromomethyl compounds. A modification of the chloromethylation procedure of Cambron (1939) was adopted for the bromomethylation of benzyl bromide, diphenyl, diphenylmethane and α:β-diphenylethane. The general method is illustrated by the following example: Diphenylmethane (39 g.) was added to a mixture of paraformaldehyde (30 g.), hydrobromic acid (77 cc., *d* 1.7), phosphoric acid (57 cc., *d* 1.75) and glacial acetic acid (95 g.). The solution was maintained at 95–110° for 5 hours while dry hydrogen bromide (20–30 g.) was passed in, left overnight and heated for a further 8 hours. The mixture was poured into water (1,000 c.c.), and the pasty precipitate filtered off and crystallized from benzene. 4:4′-*Bisbromomethyldiphenylmethane* (33 g., 40%) separated from benzene in long prisms m.p. 153.5°. (Found: C, 50.9; H, 4.0; Br, 45.1. $C_{15}H_{14}Br_2$ requires C, 51.0; H, 4.0; Br, 45.1%.)

In a similar manner were prepared p-xylylene dibromide (34% yield), 4:4′-bisbromomethyldiphenyl (35%) (*cf.* v. Braun, 1937) and 4:4′-*bisbromomethyl*-α:β-*diphenylethane* (34%) which crystallized from acetone in laminae m.p. 117–120°. (Found: C, 52.1; H, 4.5; Br, 43.1. $C_{16}H_{16}Br_2$ requires C, 52.2; H, 4.4; Br, 43.4%.)

Preparation of aromatic alkylaminomethyl derivatives (Nos. 93–106). Condensation of the bromomethyl compounds with alkylamines proceeded readily in such

solvents as alcohol, benzene or xylene, with or without the addition of sodium iodide. In all cases, however, the required product was accompanied by a considerable amount of amorphous substance of high molecular weight, apparently formed by condensation of the -NH groups in the product with further molecules of bromomethyl compound (*cf.* v. Braun, 1937); these rendered the purification of the product somewhat difficult. The formation of compounds of high molecular weight was reduced by refluxing the bromomethyl compound with an excess (10 mols.) of the alkylamine for 3–5 hours without other solvents. The excess of alkylamine was removed at 100° under reduced pressure and the residue shaken with 10% sodium hydroxide and ether. The oil obtained on evaporation of the ether was then treated with light petroleum (b.p. 40–60°) in which all the bases, with the exception of the 4 : 4′-bis(*cyclo*hexylaminomethyl) derivatives of diphenyl and α : β-diphenylethane, were very soluble, but in which the amorphous material was almost insoluble. The petroleum solution was clarified by filtration through alumina, and evaporated. The resulting base was dissolved in ethanol and the solution, filtered through kieselguhr to remove further traces of amorphous material, was treated with hydrochloric acid (*d* 1.2) to precipitate the *dihydrochloride*. This procedure was used in the preparation of all the compounds in Table III with the exception of two prepared in presence of a solvent. The *bases* were redistilled or, in some cases, crystallized from aqueous ethanol from which they separated in laminae. The dihydrochlorides were insoluble in ethanol but dissolved fairly readily in warm water to a neutral solution.

TABLE III

Compound	No.	b.p. or m.p.	Found %				Formula	Requires %			
			C	H	N	Cl		C	H	N	Cl
1 : 4-*Bis*(n-*propylaminomethyl*)*benzene*	93	b.p. *ca.*310°	76.5	10.9			$C_{14}H_{24}N_2$	76.3	11.0		
do. *dihydrochloride*		subl. > 320°	57.8	8.9		24.2	$C_{14}H_{24}N_2,2HCl$	57.3	8.9		24.2
1 : 4-*Bis*(n-*butylaminomethyl*)*benzene*	94	Oil					$C_{16}H_{28}N_2$				
do. *dihydrochloride*		subl. > 320°	60.2	9.1	8.4		$C_{16}H_{28}N_2,2HCl$	59.8	9.4	8.7	
1 : 4-*Bis*(n-*amylaminomethyl*)*benzene*	95	b.p.150°/0.2mm.	78.2	11.6	10.1		$C_{18}H_{32}N_2$	78.2	11.7	10.1	
do. *dihydrochloride*		decomp. > 320°	62.3	10.0		20.4	$C_{18}H_{32}N_2,2HCl$	61.9	9.8		20.3
4 : 4′-*Bis*(n-*butylaminomethyl*)*diphenyl*	96	m.p.43.5–45°	81.6	10.0			$C_{22}H_{32}N_2$	81.4	9.9		
do. *dihydrochloride*		decomp. > 360°	66.6	8.5		17.7	$C_{22}H_{32}N_2,2HCl$	66.5	8.6		17.8
4 : 4′-*Bis*(cyclo*hexylaminomethyl*)*diphenyl*	97	m.p.94.5–95.5°	83.8	9.8			$C_{26}H_{36}N_2$	82.9	9.6		
do. *dihydrochloride*		decomp. > 360°	69.3	8.8		15.6	$C_{26}H_{36}N_2,2HCl$	69.5	8.5		15.8
4 : 4′-*Bis*(n-*butylaminomethyl*)*diphenyl-methane*	98	m.p.9–10°	80.9	10.4	8.2		$C_{23}H_{34}N_2$	81.6	10.1	8.3	
do. *dihydrochloride*		decomp. > 320°	67.4	8.8	6.6	17.0	$C_{23}H_{34}N_2,2HCl$	67.2	8.8	6.8	17.2
4 : 4′-*Bis*(n-*amylaminomethyl*)*diphenyl-methane*	99	b.p.238°/1.0mm.	81.4	10.6	7.6		$C_{25}H_{38}N_2$	81.9	10.5	7.6	
do. *dihydrochloride*		decomp.*ca.*327°	68.8	9.1			$C_{25}H_{38}N_2,2HCl$	68.3	9.2		
4 : 4′-*Bis*(cyclo*hexylaminomethyl*)*diphenyl-methane*	100	m.p.37–38.5°	83.8	9.8			$C_{27}H_{38}N_2$	83.0	9.8		
do. *dihydrochloride*		decomp. > 320°	70.2	8.9		15.1	$C_{27}H_{38}N_2,2HCl$	70.0	8.7		15.3
4 : 4′-*Bis*(*benzylaminomethyl*)*diphenylmethane*	101	Oil	85.3	7.7	7.1		$C_{29}H_{30}N_2$	85.7	7.4	6.9	
do. *dihydrochloride*		decomp. > 350°	72.0	6.7		14.4	$C_{29}H_{30}N_2,2HCl$	72.6	6.7		14.8
4 : 4′-*Bis*(n-*propylaminomethyl*)-α : β-*diphenylethane*	102	m.p.53–54.5°	81.8	10.1			$C_{22}H_{32}N_2$	81.4	9.9		
do. *dihydrochloride*		m.p.*ca.*345°	66.5	8.4		18.0	$C_{22}H_{32}N_2,2HCl$	66.5	8.6		17.8
4 : 4′*Bis*(n-*butylaminomethyl*)-α : β-*diphenylethane*	103	m.p.46–47°	81.8	10.4	7.9		$C_{24}H_{36}N_2$	81.8	10.3	8.0	
do. *dihydrochloride*		decomp. > 320°	67.9	9.1		16.7	$C_{24}H_{36}N_2,2HCl$	67.8	9.0		16.7
4 : 4′-*Bis*(n-*amylaminomethyl*)-α : β-*diphenylethane*	104	m.p.7–8°	82.3	10.6	7.4		$C_{26}H_{40}N_2$	82.1	10.6	7.4	
do. *dihydrochloride*		decomp.333°	68.9	9.0		15.6	$C_{26}H_{40}N_2,2HCl$	68.9	9.3		15.6
4 : 4′-*Bis*(n-*heptylaminomethyl*)-α : β-*diphenylethane*	105	m.p.35–36°	81.3	11.3			$C_{30}H_{48}N_2$	82.5	11.1		
do. *dihydrochloride*		decomp.341°	70.7	10.5		13.9	$C_{30}H_{48}N_2,2HCl$	70.7	9.9		13.9
4 : 4′-*Bis*(cyclo*hexylaminomethyl*)-α : β-*diphenylethane*	106	m.p.115–116°	83.1	10.2			$C_{28}H_{40}N_2$	83.1	10.0		
do. *dihydrochloride*		decomp. > 360*	69.7	9.4		14.7	$C_{28}H_{40}N_2,2HCl$	70.4	8.9		14.8

*By refluxing 4 : 4′-bischloromethyldiphenylmethane (7.7 g.) with *n*-butylamine (7.0 g.) and sodium bromide in xylene.
†By refluxing 4 : 4′-bisbromomethyldiphenylmethane (9.7 g.) with benzylamine (7.0 g.) and sodium iodide in xylene.

Summary

1. A series of tertiary aromatic diamines in which two aromatic rings are connected by alkylene chains or chains interrupted by ether groupings have been shown to have no significant amoebicidal activity either *in vitro* or *in vivo*.

2. Some of the similarly constituted secondary diamines showed a slight activity *in vitro*.

3. Most of the secondary araliphatic diamines tested showed some activity *in vitro*, but no activity *in vivo* except at toxic dose-levels.

4. Quaternary ammonium salts were slightly more active than the corresponding tertiary bases.

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THE TREATMENT OF "SHOCK" WITH SODIUM SALT SOLUTIONS

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Rosenthal (1942–5) has stated that the administration of large quantities of sodium salt solutions reduces the mortality in mice suffering from shock caused by thermal burns, trauma, and haemorrhage. Fox (quoted by Rosenthal, 1943) in man, Allen (1943) using dogs, and Prinzmetal *et al.* (1943) in mice, have also reported good results from the use of sodium chloride solutions for the treatment of shock due to thermal burns, while Cullumbine and Box (1946) have found that large quantities of sodium chloride or sodium lactate solution reduce the mortality from lewisite shock, but that different species are not equally responsive. These results have now been confirmed in mice suffering from shock caused by thermal burning and extended to rats, rabbits, and goats burnt with liquid mustard gas.

Thermal Burning

Mice, weighing 18–20 g. and under nembutal anaesthesia, were used and their hind limbs immersed for 30 sec. in water which was maintained at a temperature of 80° C. Therapies were given at various time intervals after the immersion. The time of death after immersion for each mouse was carefully noted. Death occurred rapidly (2–4 hours) in 100 per cent of untreated mice and an effort was made to find therapies which would significantly prolong the median survival time. The results were analysed by means of Fisher's method of analysis of variance for Latin squares.

Therapy with sodium chloride.—Table I details the results and conclusions of a typical experiment. Further experiments showed that therapies administered 30 to 60 min. after burning produced better survival times than therapies administered immediately or 120 min. after burning. In general, 0.45 per cent (w/v) NaCl solution was better than a 0.9 per cent solution which, in turn, was better than a 1.8 per cent solution. All these salt solutions were better than water. Similar results were obtained whether the sodium chloride solution was given by mouth or by intraperitoneal injection.

From the results of the above experiments, it was concluded that, of the therapies tried, 1 ml. doses of the sodium chloride solutions administered 30 min. after burning gave the best chance of survival. Therefore these therapies were assessed in less severely burned mice and the percentage mortalities of the treated and untreated groups compared.

The hind limbs of mice, under nembutal anaesthesia, were immersed for 30 sec. in water maintained at 70° C. Sodium chloride solutions were administered

TABLE I

EFFECT OF INTRAPERITONEAL INJECTION OF DIFFERENT CONCENTRATIONS OF SODIUM CHLORIDE IN AQUEOUS SOLUTION ON THE SURVIVAL TIME OF MICE SUFFERING FROM THERMAL BURNS

Therapy given 30 min. after burning	Median survival time in hours (5 mice per group)	Conclusions
1 ml. water	10.0	(1) In general 1 ml. dosages are better than 2 ml. dosages in prolonging survival time. ($P = 0.05$).
2 ml. ,, ...	3.2	
1 ml. 1.8% NaCl	9.9	
2 ml. 1.8% NaCl	3.8	(2) 0.45% NaCl is better than 0.9% NaCl which in turn is better than 1.8% NaCl or water.
1 ml. 0.9% NaCl	13.5	
2 ml. 0.9% NaCl	9.5	
1 ml. 0.45% NaCl	20.0	(3) All therapies but 2 ml. water and 2 ml. 1.8% NaCl produced a significant ($P = 0.05$) increase in median survival time.
2 ml. 0.45% NaCl	15.2	
Nil	2.5	

intraperitoneally 30 min. later and the resultant total mortalities noted. These mortalities were as follows:

Untreated mice	– 24/25
1 ml. of 1.8 per cent NaCl	– 11/25
1 ml. of 0.9 per cent NaCl	– 9/25
1 ml. of 0.45 per cent NaCl	– 6/25

Hence all these therapies produced a significant reduction of the mortality, and the results agree substantially with those of Rosenthal.

Therapy with carbohydrate and protein.—It has been noted that, after thermal burns, the carbohydrate stores (liver and muscle) quickly become exhausted (Clark and Rossiter, 1944) and a large negative nitrogen balance soon occurs. (Cuthbertson *et al.*, 1939; Clark, Peters and Rossiter, 1945; Taylor *et al.*, 1943). Therefore the effectiveness of the early administration of aqueous solutions of glucose and of protein hydrolysate (a commercial preparation made by the enzymic digestion of casein) was assessed in experiments similar to those just described.

The therapies were: 5 and 10 g. glucose/100 ml. H_2O; 2.5 and 5 g. protein hydrolysate/100 ml. H_2O; the solutions were given intraperitoneally or by mouth, in 1 ml. or 2 ml. doses and at Z, Z+30, Z+60 or Z+120 min. The median survival time was not significantly prolonged by these procedures and usually it was reduced. Clark and Rossiter (1944) also found that carbohydrate feeding did not lessen the mortality among experimentally burned animals.

Mustard Gas Intoxication

The literature on human cases of mustard gas intoxication does not contain any clear-cut evidence as to the existence of a "shock syndrome" in this condition. Hermann (1918) has reported a condition of "shock" in some of the severe cases which he saw, but most accounts are silent on this subject. In animals (goats, dogs, rats) Cameron (1941) has described a haemoconcentration (as evidenced by increased haemoglobin and red blood cell estimations) in the first 24–48 hours after skin contamination; the changes were less marked in the rabbit. Cameron and Courtice (1942) have also described the later appearance of persistent diarrhoea and salivation leading to dehydration, loss of weight, and circulatory failure. Homer-Smith *et al.* (by communication in 1943), in a study on the toxic action of the β-chloroethyl vesicants in dogs, have also remarked that death is due to "anoxia" of the respiratory centres owing to peripheral circulatory failure brought on by a reduction in blood volume, which is in turn attributable to the loss of protein, electrolyte, and water through vomiting and diarrhoea, supplemented by a loss of red cells through unidentified channels. The total circulatory plasma protein is reduced, but more water than protein is lost, so that the concentration of plasma protein rises. There is a reduction in the extra-cellular fluid, circulating plasma volume, plasma chloride, and body weight; the total circulatory red cell volume is variably reduced. As the investigators have pointed out, the

> "progressive, unarrestable, and irremediable oligaemia with irreversible circulatory failure presents a close parallel to the sequence observed in dogs and man suffering fatal fire burns, haemorrhage and other forms of shock, and it is to be anticipated in men suffering severe vesication from mustard gas burns of the skin, and in whom a variable degree of systemic intoxication is superimposed on the loss of protein, the electrolytes and water through the vesicated areas."

In view of these reports of early haemoconcentration and later dehydration and electrolytic loss in mustard gas contaminated animals, it was decided to assess the value of sodium chloride administration in such animals. Three species have been studied, viz., the rat, rabbit, and goat.

Rats.—Rats (*ca.* 150 g.) were used first as the test animals; in all experiments they were contaminated by applying 10 mg./kg. of mustard gas in 1 per cent (w/v) alcoholic solution to the shaven area of the back. Precautions were taken to prevent the rats licking the contaminated area and ingesting the contaminant. The results are given in Table II.

We can conclude that in mustard gas contaminated rats:

(i) Sodium chloride solutions are an effective treatment and the sooner they are administered the better.

(ii) Although the difference is not statistically significant, sodium chloride is probably more effective when given by mouth than when given intraperitoneally.

(iii) Sodium lactate is ineffective either by mouth or intraperitoneally. (This is contrary to our experience in the treatment of lewisite intoxicated animals, and to Rosenthal's results with severely burnt mice. It emphasizes the possibility that different biochemical reactions may be involved.)

(iv) Water produced no significant effect, which suggests that electrolyte loss is a more important factor than mere dehydration in the pathology of mustard gas poisoning.

Rabbits.—Rabbits were contaminated with 150 mg./kg. of mustard gas (pure) on the shaven area of the back; licking of the area was prevented. Half the rabbits were treated with 150 ml. (given in two doses

TABLE II

EFFECT OF SODIUM SALT SOLUTIONS ON THE SURVIVAL OF RATS INTOXICATED WITH MUSTARD GAS

Treatment	Time of administration after contamination	Route	No. of animals	No. of deaths in 21 days	Per cent mortality
Nil	—	—	70	56	80
15 ml. 0.9% NaCl	At once	I.P.	20	6	30
15 ml. 0.9% NaCl	15 minutes	I.P.	20	5	25
15 ml. 0.9% NaCl	30 ,,	I.P.	20	12	60
15 ml. isotonic sodium lactate ..	15 ,,	I.P.	10	9	90
15 ml. water	15 ,,	Oral	10	7	70
15 ml. 0.9% NaCl	15 ,,	,,	20	6	30
1 ml. 15% NaCl	15 ,,	,,	10	2	20
1 ml. 30% NaCl	15 ,,	,,	10	2	20
15 ml. isotonic sodium lactate ..	15 ,,	,,	10	8	80

(I.P. = intraperitoneal)

This table shows the mortality rate in untreated rats to be 80 per cent and in rats treated with sodium chloride 37 per cent (significant at P = 0.000,000,1).

of 75 ml. at 2 and 4 hours after contamination) of 0.9 per cent NaCl by mouth; the other half were not treated. The 28-day mortalities were:

Treated rabbits — 2/10—i.e., 20 per cent.
Untreated rabbits — 7/10—i.e., 70 per cent.

This reduction in mortality is significant at P=0.04, if we take into account that the difference is in the right direction. The incidence and severity of diarrhoea and salivation and the loss of weight were much less in the treated than in the untreated group.

Goats.—The shaven backs of goats (*circa* 30 kg.) were contaminated with 35 mg./kg. mustard gas. The 0.9 per cent NaCl solution was given subcutaneously or by mouth, 1 litre at 2 hours and 1 litre at 4 hours after contamination. The 28-day mortalities for the goats in the various groups were: 7/10 in untreated goats, 7/10 in goats receiving NaCl subcutaneously, and 6/10 in goats given the NaCl by mouth. Hence the administration of sodium chloride did not reduce the mortality from mustard gas contamination in goats. Given subcutaneously, the saline solution caused massive pulmonary oedema in some of the goats (4/10), whereas by mouth it led to an increased incidence of diarrhoea (5/10, cf. 2/10 in the untreated group).

Therefore, the order of efficacy of sodium chloride solutions in the treatment of the oligaemia of mustard gas intoxication in the three species tested descends as follows: rats, rabbits, and goats. This is also, in our experience, the descending order of the ability of the different species to tolerate large quantities of sodium chloride solution.

Summary

In confirmation of previous workers, it has been found that sodium chloride solutions increase the survival time and reduce the mortality rate of mice suffering from shock induced by immersion in hot water. The best results were obtained by giving 1 ml./0.45 per cent (w/v) sodium chloride solution 30 min. after burning mice weighing about 20 g. Glucose and protein hydrolysate solutions did not increase the survival time in our experiments.

These results suggested the use of sodium salt solutions in the treatment of mustard gas intoxication. This form of therapy was highly effective in rats and rabbits; it was ineffective in goats.

The work on mustard gas intoxication was done at the Chemical Defence Experimental Station, Porton, and I am indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish these results.

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THE CONSTITUTION OF PHOTOSTILBAMIDINE, THE IRRADIATION PRODUCT OF STILBAMIDINE

BY

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Stilbamidine (4 : 4′-diamidinostilbene β-hydroxyethanesulphonate) has proved an active therapeutic agent in a number of protozoal diseases. During the treatment of Gambian sleeping sickness, Bowesman (1940) noted that solutions which had been made up for more than six hours were more toxic than those freshly prepared. Kirk (1942) suggested that the poisonous effects noted by him during the treatment of kala-azar in the Sudan were due to the use of old solutions of stilbamidine which had become toxic. Fulton and Yorke (1942) investigated the matter experimentally and showed that the increased toxicity was the result of photochemical change in the stilbamidine molecule. Similar changes were observed by Fulton (1943) in other diamidines with an unsaturated linkage. The saturation of the ethylene linkage under these conditions was indicated by the spectrographic observations of Goodwin (1943). Barber, Slack, and Wien (1943) came to the conclusion, without rigorous proof, that the irradiation product from stilbamidine was 4 : 4′-diamidinophenylbenzylcarbinol formed by the addition of water to the ethylenic bond. Henry (1943) suggested, as an alternative to carbinol formation, a number of other changes which may occur in solutions of stilbamidine exposed to light including dimerization, partial or complete hydrolysis of the amidine groups, and formation of *cis*-stilbamidine. The two latter suggestions could not be confirmed by Fulton and Goodwin (1946). In a later paper the same author (Henry, 1946) stated that aqueous solutions of *trans*-4 ; 4′-diamidinostilbene undergo two concurrent changes—dimerization and *cis-trans* isomerism—when irradiated in tropical sunlight. The proof of the dimeric nature of the saturated compound formed was arrived at from the kinetics of the photochemical reaction. The nature of this saturated compound has now been investigated. Ciamician and Silber (1902) described the formation of a dimer from stilbene when exposed for two and a half years to sunlight in benzene solution. Their work was confirmed by Stobbe (1914) who showed that the dimer could be isolated after a few days' exposure to sunlight. We have obtained the dimer described by them by irradiating stilbene in benzene with the light of a mercury vapour lamp and have used it as a reference compound to prove that the saturated product obtained from stilbamidine by irradiation is 1 : 2 : 3 : 4-tetra-(4′-amidinophenyl)-*cyclo*butane, which we have, for simplicity, called photostilbamidine.

Our thanks are due to May and Baker, Limited, for the gift of 50 g. stilbamidine with which these experiments were conducted.

Experimental

In the preparation of photostilbamidine, *trans*-4 : 4′-diamidinostilbene β-hydroxyethanesulphonate (50 g.) in 1 per cent (w/v) solution in distilled water was exposed in 500 c.c. volumes in pyrex conical flasks to direct sunlight. When freshly prepared, the solutions, before irradiation, gave rise immediately to a bulky precipitate on mixing with an equal volume of dilute sulphuric acid. After exposure of the solutions for 2 days, precipitation with sulphuric acid no longer occurred at once. At the end of one week's exposure to sunlight an equal volume of 2*N*-sulphuric acid was added to the contents of the flasks and on standing a crystalline precipitate separated. The yield of this first fraction after drying in air was 33 g. The solution was concentrated to 1,800 c.c. under reduced pressure at a temperature below 50° C., and on standing a further crop of crystals (1.9 g.) was obtained. The solution was partly neutralized with anhydrous sodium carbonate, and mixed with 600 c.c. of a saturated solution of sodium picrate ; a bulky precipitate containing sulphate ions was formed which was collected, air dried, and triturated with 16 per cent aqueous hydrochloric acid. The free picric acid was collected and washed with hydrochloric acid. The combined filtrates were extracted with ether, and heated with norite to remove all traces of picric acid. A further

2.0 g. of photostilbamidine as sulphate was obtained on concentration of the solution. The absorption spectra of the various crystalline fractions and solutions were obtained during the course of the preparation. Apart from the presence of small amounts of unchanged stilbamidine, amounting to approximately 5 per cent of the original, the only other substance detected was photostilbamidine. The latter, as sulphate, was purified for analysis by recrystallization from water in which it was sparingly soluble; it crystallized in short rods with terminal branching, and melted between 280 and 290°. Purified *photostilbamidine sulphate* in solution showed only the faintest fluorescence in ultra-violet light in contrast to the marked fluorescence of solutions of stilbamidine. The air-dried product was analysed (Found: C, 44.5; H, 6.2; N, 12.6; H_2O, 15.7. $C_{22}H_{32}N_8$, $2H_2SO_4$, $8H_2O$ requires C, 44.2; H, 6.0; N, 12.9; H_2O, 16.6%). Photostilbamidine sulphate dissolved in water and treated with the theoretical amount of barium chloride gave a very soluble crystalline dihydrochloride. When the solution of the latter was mixed with ammonium nitrate in excess, a crystalline *nitrate* was deposited. Recrystallization from water gave compact crystals, m.p. 288° (decomposition). (Found in air-dried solid: C, 46.6; H, 5.3; N, 20.1; H_2O, 5.7. $C_{22}H_{32}N_8$, $4HNO_3$, $3H_2O$ requires C, 46.0; H, 5.1; N, 20.1; H_2O, 6.5%.)

Hydrolysis of stilbamidine and photostilbamidine sulphate

Photostilbamidine sulphate (1.3 g.) was mixed with potassium hydroxide (5 g.), water (5 c.c.), and absolute alcohol (45 c.c.), and boiled for 22 hours with the addition of water (15 c.c.) at the start of each day to dissolve some of the salts present. An equal volume of water was then added to the mixture and the alcohol distilled off. After removal of a small amount of insoluble material, the filtrate was acidified and yielded a gelatinous precipitate which became granular when heated on the water bath. It was collected, thoroughly washed with water, and redissolved in sodium bicarbonate solution; the solution was filtered from inorganic material and acidified. The product was again dissolved in aqueous sodium bicarbonate and reprecipitated. The yields of acid in two experiments each from 6.7 g. of starting material were 3.80 g. and 3.85 g., after drying at 90°. The product was nitrogen-free and required the theoretical amount of base for neutralization. On crystallization from glacial acetic acid, a small amount of insoluble material, possibly stilbenedicarboxylic acid, was removed. After three recrystallizations, 0.39 g. of the product required 35 c.c. of boiling glacial acetic acid for solution and gave 0.28 g. of a colourless product. Two forms of crystal were present, a needle-shaped variety being preponderant with some hexagonal plates. The *tetra-acid* was also soluble in ethyl alcohol, acetone and ethyl acetate and did not melt below 310°. (Found in solid dried at 90°: C, 69.4; H, 5.1; loss at 110°, 3.1. $C_{32}H_{24}O_8$, H_2O requires C, 69.3; H, 4.7; H_2O, 3.2%.) The *methyl ester* was prepared by the action of an excess of diazomethane in ether on 0.1 g. of purified acid in 10 c.c. of methyl alcohol; after leaving overnight a clear solution was obtained, and when the solvent was removed an oil separated which soon solidified to a colourless solid. It crystallized from methyl alcohol in very small plates; m.p. 166°. (Found in air-dried material: C, 72.9; H, 5.4. $C_{36}H_{32}O_8$ requires C, 73.0; H, 5.4%.)

The hydrolysis of stilbamidine was found to be a much more difficult process. When 4:4′-diamidinostilbene dihydrochloride (2.5 g.), potassium hydroxide (10 g.) in water (10 c.c.) and absolute alcohol (90 c.c.) were refluxed for 20 hours in the dark, only 130 mg. of an acid were obtained by the procedure described above. In another experiment the same material (2.5 g.), sodium hydroxide (5 g.) in water (10 c.c.) and ethylene glycol (40 c.c.) were refluxed for 9 hours. The insoluble material present was removed and the filtrate concentrated under reduced pressure; the sodium salt obtained was collected, redissolved in water, and by the usual procedure yielded 0.54 g. acid, which was nitrogen-free, was insoluble in all common organic solvents and could not be crystallized. The ammonium salt, however, was very characteristic and crystallized readily in sheaves of leaflets. The solid first collected after alkaline hydrolysis weighed 1.57 g. and contained nitrogen. Hydrolysis of this material was effected by refluxing with 60 per cent sulphuric acid (25 c.c.) for 7 hours and then for a further 7 hours after addition of an equal volume of sulphuric acid of the same strength. On cooling the mixture was poured into water, the solid collected, and after purification by solution in sodium bicarbonate solution the yield of air-dried material obtained on acidification was 0.9 g. and had similar properties to the product of alkaline hydrolysis.

Esterification could not be satisfactorily effected by refluxing the acid (0.5 g.) with methyl alcohol (20 c.c.) and concentrated sulphuric acid (2 c.c.) on the water bath for 4 days. The desired product was, however, obtained by allowing suspensions of the acid, from both alkaline and acid hydrolysis, in methyl alcohol and ether to react with diazomethane. The bulk of the reaction product was very sparingly soluble and separated as small rhomboidal plates; a further small amount was obtained by concentration of the mother liquors. This methyl ester was very sparingly soluble in boiling methyl alcohol but crystallized well from dioxane in glistening fasciated laths; the addition of methyl or *iso*propyl alcohol to this solvent favoured quantitative separation. The ester from both products had m.p. 234°. Meyer and Hofmann (1917) gave the m.p. of dimethyl *pp′*-stilbene-dicarboxylate as 226–227° and Hager *et al.* (1944) recorded the m.p. as 227–228°.

Decarboxylation of 1:2:3:4--tetra-(p-carboxyphenyl)-cyclobutane. Isolation of distilbene, isodistilbene and stilbene

The tetracarboxylic acid (0.5 g.) was mixed with quinoline (10 c.c.) and copper bronze (0.5 g.) and the

mixture boiled for 7 hours on a sand-bath. Evolution of carbon dioxide was slow at first but soon increased as the acid dissolved. On cooling, the mixture was poured into excess of 2*N*-hydrochloric acid and extraction of the product was effected by several portions of ether. The combined ether extracts were washed in turn with *N*-hydrochloric acid, sodium bicarbonate solution, and finally water. On removal of the solvent, 0.30 g. of a crystalline material was obtained. It was recrystallized from ether and separated as a mixture of fine prisms and colourless plates. The experiment was repeated with 1.5 g. acid, yielding 1.0 g. of product, which when refluxed with ether did not all go readily into solution. The less soluble portion was collected separately and crystallized from this solvent in the form of fine prisms, m.p. 163°. (Found: C, 93.3; H, 6.7. Calc. for $C_{28}H_{24}$: C, 93.3; H, 6.7%. M.W. in camphor, 292. Calc., 360.) The properties of this hydrocarbon agree with those described for distilbene by Ciamician and Silber and it must be 1:2:3:4-tetraphenyl*cyclo*butane. The more soluble portion separated on concentration of the first mother liquors, as colourless plates. They were shown by m.p. and mixed m.p. with a pure specimen to be stilbene. The yield of stilbene was approximately 1/5 that of the other product. When decarboxylation under the same conditions was continued for 10 hours with 3.0 g. acid obtained from carefully purified photostilbamidine sulphate the product weighing 2.15 g. was highly coloured and contained a small amount of tarry material. It was also much more soluble in ether, and from this solvent large crystals of stilbene separated first. The only other product obtained was that melting at 163°. It seemed that some decomposition had occurred during this longer decarboxylation and stilbene was present in relatively greater quantity than in the preceding experiment. A final decarboxylation with similarly purified material (1.0 g.) lasting 4½ hours yielded 0.68 g. of product. By fractional crystallization from ether a large crop of the product m.p. 163° was obtained. The next fraction yielded hexagonal plates which when crystallized from ether or methyl alcohol melted at 149° in a total yield of approximately 70 mg. The only other product obtained was a few milligrams of stilbene. It appeared, therefore, that the yield of stilbene increased with increase of the reaction time, and the hydrocarbon m.p. 149° could not be isolated when the reaction was continued for more than than 4½ hours. The new hydrocarbon could be crystallized from ether or methyl alcohol and separated from the latter solvent in small tablets. As it is isomeric with distilbene, the name iso*distilbene* is proposed for it. (Found: C, 92.7; H, 6.8. $C_{28}H_{24}$ requires C, 93.3; H, 6.7. M.W. in camphor, 307. Calc., 360.)

Stilbene-4:4′-dicarboxylic acid on decarboxylation in the presence of quinoline and copper bronze for 4½ hours gave a theoretical yield of hydrocarbon. Careful fractionation from ether and absorption on alumina failed to reveal any other hydrocarbon than stilbene. When stilbene and the hydrocarbon m.p. 163° were respectively heated with quinoline and copper under the same conditions, each substance was recovered unchanged from the reaction mixture.

Fractionation of the acid product from photostilbamidine

In order to throw light on the presence of stilbene obtained along with the hydrocarbon m.p. 163° in all the decarboxylation experiments, the photostilbamidine used in the preparation of the acid was examined for purity. In water it showed fluorescence similar to that of a weak solution of stilbamidine and spectrophotometric determinations indicated that about 5 per cent of this substance was present. Accordingly 14.4 g. of the original photostilbamidine was recrystallized from water and 0.62 g. of very sparingly soluble material with all the properties of stilbamidine sulphate, including characteristic absorption spectrum, was collected. Further light on the homogeneity of the original photostilbamidine was sought by an examination of the acid obtained from it. This acid was subjected to a fractionation process depending on the different acidities of the acids (*cf.* King and Ware, 1941). For this purpose 3.62 g. of acid were accurately neutralized with 27 c.c. of approximately *N*-sodium hydroxide in a total volume of about 150 c.c. of aqueous solution. The solution was then saturated with ether and a layer of 50 c.c. of the same solvent added. 2.7 c.c. of N-hydrochloric acid was then added slowly with shaking. Part of the precipitated acid in this first fraction remained undissolved by the ether. Ten further similar volumes of hydrochloric acid were added in turn, and the liberated acid dissolved in the freshly added ether layer in each case on shaking. After removal of solvent the weights of acid in grams obtained from each fraction in turn were as follows: 0.05 (insoluble) plus 0.06 (soluble); 0.17; 0.33; 0.40; 0.50; 0.45; 0.44; 0.44; 0.44; 0.15. All the fractions, in methyl alcohol, were methylated by means of diazo-methane. From each, the methyl ester already described of m.p. 166° was obtained. Only the insoluble part of the first fraction yielded the methyl ester of stilbenedicarboxylc acid, m.p. 234°. The amount of this acid present was therefore probably less than 2 per cent and this amount failed to explain the presence of stilbene in the relatively large amounts found after decarboxylation.

Irradiation of Stilbene

In contrast to the rapidity and completeness with which photochemical change occurs in stilbamidine solutions, the reaction in stilbene solutions occurs relatively slowly and incompletely. The formation of a distilbene m.p. 163° was described by Ciamician and Silber (1902) who exposed stilbene, in benzene solution, for 2½ years to sunlight. The same product was obtained by Stobbe (1914) after 3 days' exposure, but the percentage yield obtained was not indicated

by any of these authors. We exposed 1 g. of stilbene in 10 c.c. of pure benzene to sunlight (not intense) for six weeks in a sealed tube but failed to isolate any of the dimerization product. The same solution was further exposed in a quartz cell 1 cm. deep to light of wavelength 250 mμ for 4 hours again without success. On further irradiation with the light from a mercury vapour lamp for 14 hours, the solvent was removed *in vacuo* and the crystalline residue redissolved in ether which was then concentrated. Fine prisms weighing 60 mg., readily distinguishable from the plates characteristic of stilbene, were the first to separate and a further smaller crop of prisms was obtained. On purification from ether the prisms melted at 163°, and from their properties, including stability to permanganate, non-absorption of bromine and unchanged melting point on admixture, appeared to be the same substance as that described by the above authors. There was no change in melting point on admixture with the decarboxylation product m.p. 163° described above. The substance produced by the irradiation of stilbene and the decarboxylation product were shown to be identical by means of *x*-ray crystallographic analysis (Fulton and Dunitz, 1947) and by comparison of their absorption spectra (Goodwin, 1947) in *cyclo*hexane (Fig. 1). Theoretically the position of the absorption band and the intensity measured as $E^{1\%}_{1\ cm.}$ should be very similar to those for toluene, and this was found to be the case.

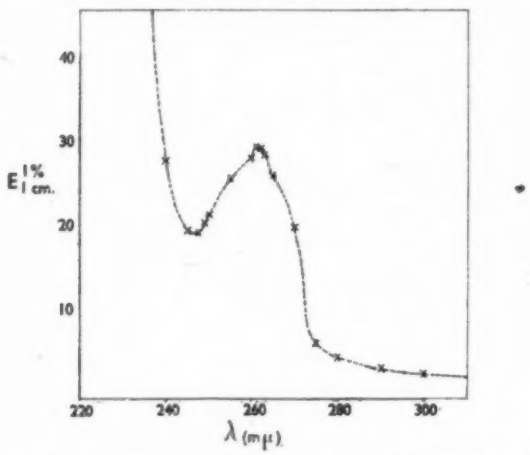

FIG. 1.—Absorption spectrum of 1 : 2 : 3 : 4-tetraphenyl-*cyclo*butane in *cyclo*hexane.

	max.	$E^{1\%}_{1\ cm.}$
Compound m.p. 163°	261 mμ	29.9
Toluene	262 mμ	32.7

When a stilbene solution in benzene was irradiated by the light of a mercury vapour lamp for 30 hours there was obtained besides unchanged stilbene and the hydrocarbon m.p. 163° a substance in yield of only a few milligrams, which crystallized in colourless needles from ether and had m.p. 213°. Analysis indicated that it contained oxygen. Because of the extremely small amount available its nature could not be determined. In another experiment in which 1.35 g. stilbene in benzene was exposed under the same conditions the yield of hydrocarbon m.p. 163° was 240 mg.

DISCUSSION

On exposure of stilbamidine solutions to sunlight the only product obtained, apart from small amounts of unchanged material, was photostilbamidine, isolated as sulphate. We now have clear chemical evidence that photostilbamidine is a dimerization product of stilbamidine. Analyses of photostilbamidine sulphate and nitrate show that the elements of water are not involved in the change of stilbamidine into photostilbamidine. When photostilbamidine was hydrolysed an acid was obtained different from stilbene-*p* : *p*′-dicarboxylic acid but of the same fundamental composition. It gave a methyl ester, m.p. 166° different from the methyl ester of stilbene-*p* : *p*′-dicarboxylate which has m.p. 234°. On decarboxylation of the acid from photostilbamidine, a hydrocarbon, $C_{28}H_{24}$, m.p. 163° was obtained identical with distilbene first isolated by Ciamician and Silber from the irradiation of benzene solutions of stilbene. A second isomeric hydrocarbon *iso*distilbene m.p. 149° was found in one decarboxylation experiment.

If during the irradiation of *trans*-stilbamidine the activated molecule retains its *trans*-structure, combination of two activated *trans*-molecules can give rise to two isomeric products which on hydrolysis and decarboxylation should yield the two tetraphenyl*cyclo*butanes A and B.

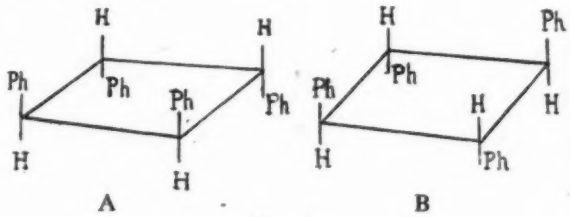

FIG. 2.

If however the activated molecule passed through or into a *cis*-form owing to free rotation, combinations involving *cis*-forms would give rise in addition to two other isomeric hydrocarbons.

It has been shown (Fulton and Dunitz, 1947) that the distilbene, m.p. 163° is represented by A with a centre of symmetry. The spatial configuration of the isomeride, *iso*distilbene, m.p. 149° has not yet been determined, but the probability is that

it has structure B. Since photostilbamidine in all experiments gave the hydrocarbon A, usually as the major component, the structure of photostilbamidine is that shown in A with four amidine groups in the *p*-positions of the phenyl groups.

Since *iso*distilbene was isolated on one occasion it is possible that photostilbamidine sulphate contains a component corresponding to structure B. The acid obtained by hydrolysis of photostilbamidine, on crystallization from acetic acid, showed inhomogeneity of crystal form and on decarboxylation under milder conditions gave two isomeric hydrocarbons apart from stilbene. On the other hand, careful fractionation of the acidic product obtained on hydrolysis of photostilbamidine failed to show the presence of any isomeric tetracarboxylic acid since all the fractions gave the same methyl ester m.p. 166°. The most likely explanation of this is that the isomeric tetracarboxylic acid which gives rise to the hydrocarbon, m.p. 149° must have been eliminated in the numerous purification stages involved in the preparation of the tetra-acid from photostilbamidine before subjection to decarboxylation.

The formation of stilbene in the decarboxylations of the tetra-acid is definitely not due to the presence of stilbene-dicarboxylic acid as an impurity. Stilbene arises from very specially purified photostilbamidine and its proportion increases with the duration of the decarboxylation process.

Summary

Irradiation of a solution of *trans*-stilbamidine in sunlight gave a substance which on hydrolysis to the corresponding acid, followed by decarboxylation, yielded a hydrocarbon m.p. 163° identical with that obtained by irradiation of stilbene. The hydrocarbon previously described as di-stilbene has now been shown by *x*-ray crystallographic analysis to be 1 : 2 : 3 : 4-tetraphenyl-*cyclo*butane, containing a centre of symmetry. A second isomeric hydrocarbon m.p. 149° was also obtained in the same series of reactions, but its molecular configuration has not yet been determined. The isolation of these two hydrocarbons is in agreement with the view that stilbamidine undergoes dimerization on irradiation of its solutions.

I wish to record my thanks to Dr. Harold King for generous help during the course of this investigation, and to Mr. T. W. Goodwin for the determinations of the absorption spectra.

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THE EFFECT OF INJECTED SOLUTIONS ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID

BY

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An account has been given in an earlier paper (Bedford, 1946) of the effect of the introduction of isotonic sodium chloride solution into the cisterna magna on the cell content of the cerebrospinal fluid of dogs anaesthetized with "nembutal" (Abbott). The duration of these experiments was approximately five hours. Polymorphonuclear leucocytes, generally unaccompanied by other reactive cells, were present at the end of nine out of thirteen experiments. The introduction under the same conditions of Ringer's solution (Dale's formula) or of distilled water did not cause reactive cells to appear in the cerebrospinal fluid. In the experiments now reported, the changes in cell count have been investigated 24 hours after the introduction of an isotonic solution of sodium chloride into the cisterna magna, and a comparison has been made with the effects produced after a similar period of time by solutions of procaine, amylocaine, and amethocaine hydrochlorides. A study has also been made of the effect of simple puncture of the cisterna magna, the introduction of distilled water and of Ringer's solution.

Experimental procedure

Except for slight modifications, the experimental procedure was similar to that used in earlier experiments (Bedford, 1946). In this series of experiments the animals were anaesthetized with ether. They were allowed to recover from the anaesthetic after the solution under investigation had been introduced and the needle withdrawn. It was necessary, however, after the introduction of local anaesthetics to maintain respiration with the pump until the onset of spontaneous respiration. The animals were again anaesthetized with ether twenty-four hours later and a sample of cerebrospinal fluid removed for cytological examination. The films were fixed by exposure to iodine vapour after the technique of Kubie and Smith (1925) and lightly stained with methylene blue. The use of this technique has greatly facilitated cellular differentiation. In the present series of experiments a finer needle has been used for cisternal puncture; as a result, detached mesothelial cells have been observed only on rare occasions in specimens of cerebrospinal fluid, and the possibility of post-operative leak from the subarachnoid space has been reduced. The puncture was made without previous incision directly through the shaved and sterilized skin. An experiment was discontinued if the initial sample of cerebrospinal fluid contained cells of any kind. The solutions were made up with freshly distilled water from an all-glass still. Although the individual solutions were not tested for the presence of pyrogens, repeated examination of distilled water from this still has never revealed their presence. The solutions were sterilized, without delay, in an autoclave at a pressure of 12 lb. per square inch for half an hour. The Ringer's solution was boiled for 20 min. at atmospheric pressure. Care was taken to sterilize the solutions in alkali-free glass containers. A constant volume (1.5 c.c.) of solution, at room temperature, was introduced into the subarachnoid space throughout the experiments.

RESULTS

The effect of ether anaesthesia

Four dogs were anaesthetized with ether; one for a period of 15 minutes, two for 30 minutes, and one for one hour. The ether was administered by means of a pump through a catheter, introduced into the trachea. In all four dogs, samples of cerebrospinal fluid removed 24 hours later under ether anaesthesia were free from cells.

The effect of simple puncture and of distilled water

The effect of simple puncture of the cisterna magna was studied in ten dogs. After introduction, the needle was allowed to remain *in situ* with the stilette inserted for ten minutes. At the end of this period, the needle was withdrawn and the animal allowed to recover. The results obtained 24 hours later are summarized in Table I,

TABLE I

THE EFFECT OF SIMPLE PUNCTURE OF THE CISTERNA MAGNA AND OF DISTILLED WATER ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID

Simple puncture		Distilled water (1.5 c.c.)	
Wt. of dog in kg.	White cells per cu.mm. C.S.F. after 24 hours	Wt. of dog in kg.	White cells per cu.mm. C.S.F. after 24 hours
11.0	40	10.0	480
8.0	70	9.0	450
7.5	60	8.5	450
11.0	70	10.0	345
8.0	20	7.5	420
8.6	40	6.0	400
12.5	12	—	—
7.5	40	—	—
8.0	0	—	—
6.5	12	—	—
Mean ± S.D.	36 ± 8		424 ± 19

where it will be noticed that white cells were present at the end of all but one experiment. The average number of cells per cu.mm. of cerebrospinal fluid was 36. The effect of the introduction of distilled water was studied in six animals. The results are summarized in Table I, where it will be seen that the average number of white cells at the end of the experiment was 424, and the standard deviation of the average 19.

The effect of isotonic sodium chloride and of Ringer's solution

The effect of isotonic sodium chloride solution (0.9 g. NaCl/100 c.c.) was studied in nine experiments and that of Ringer's solution (Dale's formula) in six experiments. The *p*H values of the solutions after sterilization were 6.8 and 7.4 respectively, as determined by indicators. The results of the experiments are summarized in Table II.

The effect of solutions of procaine, amylocaine, and amethocaine hydrochlorides

The effect of procaine was studied in ten experiments; five experiments were performed with

TABLE II

THE EFFECT OF ISOTONIC SODIUM CHLORIDE SOLUTION AND OF RINGER'S SOLUTION (DALE'S FORMULA) ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID AFTER 24 HOURS

Normal Sodium Chloride Solution (1.5 c.c.)		Ringer's Solution Dale's Formula (1.5 c.c.)	
Wt. of dog in kg.	White cells per cu.mm. C.S.F. after 24 hours	Wt. of dog in kg.	White cells per cu.mm. C.S.F. after 24 hours
7.5	1,000	9.0	740
5.5	750	6.0	490
8.0	1,500	8.0	720
7.0	750	7.0	690
6.0	700	7.5	470
8.5	1,500	6.5	680
7.0	800	—	—
8.5	1,600	—	—
7.5	1,000	—	—
Mean ± S.D.	1,067 ± 109		632 ± 49

amylocaine and a similar number with amethocaine. Procaine and amylocaine were administered in a 1 per cent (w/v) concentration in distilled water. The depressant action of amethocaine on respiration was so powerful and prolonged that it was found convenient to use a solution not stronger than 0.25 per cent; even after the introduction of a solution of this concentration, respiration was frequently paralysed for half an hour or longer. The *p*H values of the procaine, amylocaine, and amethocaine solutions after sterilization were 5.9, 5.9, and 6.0 respectively. The results of these experiments are summarized in Table III.

It will be seen that the average number of cells per cu.mm. of cerebrospinal fluid at the end of the experiments was approximately the same with all three drugs.

Discussion

It would seem from the above experiments that the introduction into the subarachnoid space of isotonic sodium chloride solution, procaine, and amylocaine hydrochlorides in 1 per cent (w/v) and amethocaine hydrochloride in 0.25 per cent (w/v) concentration in distilled water excites reactions of approximately equal intensity as determined by the number of white blood corpuscles in the cerebrospinal fluid after 24 hours. Ringer's solution caused a more intense reaction than distilled water, but not so powerful as that of isotonic sodium chloride solution; it occupied a position intermediate between the two. The standard deviations of the averages obtained in these experiments indicate that the above differences in

TABLE III

THE EFFECT OF PROCAINE, AMYLOCAINE AND AMETHOCAINE HYDROCHLORIDES ON THE CELL CONTENT OF THE CEREBROSPINAL FLUID

1% (w/v) Procaine (1.5 c.c.)		1% (w/v) Amylocaine (1.5 c.c.)		0·25% (w/v) Amethocaine (1.5 c.c.)	
Weight in kg.	White cells per cu.mm. C.S.F. after 24 hours	Weight in kg.	White cells per cu.mm. C.S.F. after 24 hours	Weight in kg.	White cells per cu.mm. C.S.F. after 24 hours
6.5	1,340	6.2	1,600	8.5	670
10.5	840	12.0	800	7.5	1,490
12.0	1,500	12.5	810	8.0	730
8.0	810	7.5	1,090	9.5	1,360
10.0	900	12.0	700	6.5	1,500
7.0	1,200	—	—	—	—
8.5	750	—	—	—	—
7.5	825	—	—	—	—
9.0	1,180	—	—	—	—
8.5	1,010	—	—	—	—
Mean ± S.D.	1,035 ± 81		1,000 ± 163		1,150 ± 185

intensity of reaction are statistically significant. The lack of correlation between the weight of the animal and the reaction provoked by a constant volume of solution was unexpected. It can, however, be accounted for in several ways. The dogs used in these experiments were mongrels; little selection has been possible and consequently marked variation was noticed in the size and shape of the cisterna magna of dogs of the same weight. Variations will accordingly occur in the degree of dilution of solution immediately after introduction and in the rate at which it leaves the cisterna magna. An important cause of lack of correlation between body weight and reaction to a given volume of solution is leakage through the puncture hole after withdrawal of the needle. It has been possible to demonstrate that the puncture hole through the dura and the arachnoid does not always close after withdrawal of the needle; the longer the needle remains inserted, the more likely is leakage to occur. A leaking puncture hole can produce two important results: if the pressure in the cisterna magna is high, as normally occurs when the animal is inclined towards the "head-down-tail-up" posture, fluid escapes, often at a considerable rate, from the cisterna magna into the extradural region. On the other hand, if the animal is inclined towards the "head-up-tail-down" posture, the pressure in the cisterna magna falls and aspiration may take place from the extradural region. The fluid aspirated generally consists of blood and tissue fluid, which are known (Hammes, 1944) to have a marked irritant action on the meninges. These phenomena have frequently been demonstrated on the living animal during the course of the experiments. Their occurrence can be avoided to a certain extent by the use of a fine needle and by careful avoidance of any disturbance of the needle after introduction.

The cells present in the cerebrospinal fluid at the end of the experiments consisted almost entirely of polymorphonuclear leucocytes; in many instances they were the only cells detected. Lymphocytes rarely formed more than 3 per cent of the total white cells.

It is improbable that the cellular reaction provoked by local anaesthetics is a *p*H response. Experiments *in vitro* have shown that the solutions rapidly assumed the *p*H of the cerebrospinal fluid on the addition of a relatively small volume of the latter fluid. Injected solution, withdrawn a few seconds after introduction into the cisterna magna, was generally found to have acquired the *p*H of cerebrospinal fluid.

In spite of technical difficulties, the experiments would appear to demonstrate that distilled water is a less powerful irritant to the meninges than isotonic sodium chloride solution and thereby to confirm the results of earlier experiments (Bedford, 1946), that isotonic sodium chloride solution, procaine, and amylocaine hydrochlorides in a 1 per cent (w/v) and amethocaine hydrochloride in a 0.25 per cent concentration in distilled water excite reactions of approximately equal intensity.

The results of the experiments raise doubt as to the advisability of using simple sodium chloride in order to render isotonic solutions required for introduction into the subarachnoid space.

SUMMARY

1. A study has been made of the cell content of the cerebrospinal fluid 24 hours after the introduction of procaine, amylocaine, and amethocaine into the cisterna magna of dogs and the effects of these drugs have been compared with those produced by distilled water, isotonic sodium chloride and Ringer's solution over a similar period of time.

2. Isotonic sodium chloride solution, 1 per cent procaine and amylocaine hydrochlorides and 0.25 per cent amethocaine hydrochloride in distilled water excited reactions of approximately equal intensity. Ringer's solution caused a more intense reaction than distilled water, but less than that of isotonic sodium chloride solution.

3. The results of these experiments raise doubt as to the advisability of using simple sodium chloride in order to render isotonic solutions required for introduction into the subarachnoid space.

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ACTIONS OF BRITISH ANTI-LEWISITE (2 : 3-DIMERCAPTOPROPANOL)

BY

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Peters, Stocken, and Thompson (1945) and Waters and Stock (1945) described the effectiveness of 2:3-dimercaptopropanol (named British Anti-Lewisite or BAL by American workers) in countering the effects of arsenical poisoning. Apart from the importance of having an efficient antidote to arsenical agents in chemical warfare, there arose the possibility of using BAL for the treatment of the toxic manifestations of arsenical drugs in therapeutics. After investigations of the protective action of BAL against arsenical poisons carried out in this country and at the same time those on lewisite by Harrison, Durlacher, Albrink, Ordway, and Bunting (1946) the substance was used successfully by Carleton, Peters, Stocken, Thompson, and Williams (1946) and Eagle (1946) as an antidote in clinical cases of poisoning by such therapeutic agents as mapharside.

The protective action of BAL stimulated research into the mode of action of dithiols in detoxicating arsenic. Peters, Sinclair, and Thompson (1946) and Stocken and Thompson (1946) have shown that arsenic combines with the available sulphydryl groups in proteins and in this way interferes with the enzymes vital to cellular metabolism which depend for their activity on the presence of SH-groups; one such enzyme is pyruvate oxidase. A molecule of BAL contains two SH-groups and can thus re-activate enzymes which have been inhibited by arsénic (Stocken, Thompson, and Whittaker, 1947).

The nature of the reaction between arsenic and BAL made it seem probable that BAL would react similarly with other metals, and Gilman, Philips, Allen, and Koelle (1946) and Ginzler, Gilman, Philips, Allen, and Koelle (1946) showed that this was so with cadmium. Braun, Lusky, and Calvery (1946) showed the same with antimony, bismuth, chromium, mercury, and nickel. Lead and selenium were made more toxic by BAL, while thallium was not affected.

The toxic properties of BAL were investigated by Durlacher, Bunting, Harrison, Ordway, and Albrink (1946) and Modell, Chenoweth, and Krop (1946) who stated that the LD50 in rabbits was 99 mg./kg. and in cats 0.032 ml./kg. The chief symptoms of toxicity found were conjunctivitis, gastro-enteritis, tremors, and ataxia. Fatal doses caused convulsions and circulatory and respiratory failure. According to Modell, Gold, and Catell (1946) doses in excess of 3-5 mg./kg. in man cause blepharospasm, flushes, and unpleasant sensations.

It was decided to investigate the action of BAL on the toxicity of several metallic compounds deemed to be of clinical importance, viz.—mapharside, mercuric chloride, mersalyl, potassium antimony tartrate, lead acetate, sodium bismuth tartrate, sodium auro-thiomalate and chromium trioxide. In addition the effect of BAL on the physiological action of insulin was examined. As a preliminary step the toxicity of BAL itself was investigated.

EXPERIMENTAL

Methods.—The effects of BAL were investigated by injection of freshly prepared watery solutions of the compound by various routes into mice, rats, guinea-pigs, and rabbits. Rabbits anaesthetized with 25 per cent (w/v) urethane solution and cats anaesthetized with ether and chloralose (80 mg./kg. i.v.) were used to determine the actions of BAL on the blood pressure, heart (myograph), spleen volume (plethysmograph and piston recorder), leg volume (plethysmograph and tambour), and respiration (stethographic lever). Isolated rabbit auricles, isolated perfused cat hearts, and isolated strips of gut and uterus from rabbit, cat, and guinea-pig were examined in the usual way. The vessels of the rabbit ear were perfused with saline according to the method of Gaddum and Kwiatkowski (1938), but as no recording apparatus was available the outflow was measured in drops per 15 sec. using larger doses of drugs than would otherwise be necessary. The method of investigating the effect of BAL on the diuretic activity of mersalyl was that

described by Burn (1937) for the assay of the anti-diuretic potency of extracts of the posterior pituitary body.

The LD50 of the metallic compounds was roughly determined by intra-peritoneal injection in small groups of mice; 200 male mice of 20–30 g. weight were then taken in four groups of 50 and given graded doses of the metallic compound so as to cover an adequate range of toxicity. To half of each group of 50 BAL was then given in a standard dose of 40 mg./kg. i.p. and the mortalities noted after 24 hours. Where the detoxifying action of BAL was such as to cause all the animals so protected to survive, the experiment was repeated with higher doses of metallic compound in the animals receiving protection. All injections with one metal were done at a single session. The LD50 was read from the plot of log dose and probit of lethality.

Results

Toxicity of BAL

The LD50 of BAL dissolved in water was found to be 100 mg./kg. for white mice injected intraperitoneally, which agrees well with the 0.8 mM./kg. of Durlacher *et al.* (1946). Three guinea-pigs given 150 mg./kg. died, and three given 50 mg./kg. survived; 12 rats survived 40 mg./kg. i.p., and of six rabbits given 100 mg./kg. i.v. two died.

In mice a lethal dose given intraperitoneally caused immediate weakness of the legs; analgesia was marked at this early stage, severe nipping of the tail being ignored; respiration was slow and laboured, but no tremors were seen; clonic and tonic convulsions followed, interrupted by periods of coma; death was accompanied by signs of asphyxia. Sublethal doses produced marked ataxia and weakness but not convulsions. In rabbits and guinea-pigs small doses (20 mg./kg. i.p.) produced blepharospasm and sneezing in 15 min., followed by tremor, weakness of the legs, and ataxia in 30 min. to one hour. Salivation was notable, urine and faeces were passed frequently, and the respiration was deep and hurried. If the dose was lethal the tremor increased to generalized convulsions, respiration was markedly impaired, and death occurred in tonic convulsion. *Post mortem* the liver, spleen, kidneys, and gut were congested and the lungs covered with small haemorrhages. Section of the lungs revealed haemorrhagic exudates.

Circulatory and respiratory effects

In rabbits anaesthetized with 25 per cent (w/v) urethane, BAL in a dosage of 0.5–1.0 mg./kg. in saline had no appreciable effect on the blood pressure or respiration, whereas 4.0 mg./kg. caused a sharp rise in blood pressure of some 20–30 mm.Hg and a stimulation of respiration which was maintained for several minutes; this effect of small doses of BAL on the circulation and breathing in the rabbit is illustrated in Fig. 1. Larger doses (20–40 mg./kg.) caused a transient rise in blood pressure and stimulation of respiration which was followed by a steady decline in pressure and failure of breathing. If the lethal dose was approached this course might be interrupted by convulsions, though usually the blood pressure declined to zero and the animal died

FIG. 1.—Rabbit ♂ 2.4 kg. Urethane 25 per cent i.v. and ether. Upper record carotid blood pressure, lower record respiration (stethograph, inspiration down). Time in 30 secs. BAL (4.0 mg./kg. i.v.) causes a rise in blood pressure and stimulation of respiration.

quietly after a few final gasps. This was in contrast to the mode of death of the unanaesthetized animal which convulsed violently, but the lethal dose was no smaller in the unanaesthetized animal, nor was the toxic effect modified by atropine sulphate, vagotomy, or artificial respiration. The isolated auricles of the rabbit continued to beat well in the presence of BAL in high concentration (1 in 10,000). The perfused vessels of the rabbit ear were constricted by BAL. Single injections of 3.0 mg. BAL in saline caused sharp but very transient vaso-constriction; constant perfusion with BAL (1 in 10,000) produced a reduction in flow.

In cats anaesthetized with ether, chloralose or nembutal, intravenous BAL invariably caused an abrupt fall in blood pressure accompanied by transient shrinkage of the spleen. If 20 mg./kg. was given the blood pressure fell abruptly and was quickly but usually only partially restored,

though sometimes it rose again above the initial level for a few minutes. The abrupt fall in pressure was accompanied by shrinkage of the spleen, or sometimes by dilatation ; the phase of recovery, partial or complete, was accompanied by active splenic dilatation. The leg was constricted and the heart unchanged in vigour and amplitude of beat so that splanchnic dilatation would appear to account for the early stages of the fall in pressure. The relative degree of alteration in flow in the splanchnic and limb circulations accounts for the varying responses of the blood pressure. Respiration was stimulated. Within a few minutes a progressive fall in blood pressure and inhibition of respiration ensued. Neither the abrupt nor the progressive fall in blood pressure was prevented by atropine sulphate (1.5 mg./kg.), by bilateral vagotomy or by artificial respiration, so that there is no question of vagal inhibition playing a part in the initial fall in pressure nor of failure of oxygenation of the blood playing a part in the later fall in pressure. After a few minutes, while the blood pressure was steadily falling, the spleen contracted maximally. The blood pressure at this stage could be temporarily restored by injection of adrenaline or infusion of saline. The restorative effect of saline soon passed off, blood pressure fell progressively, the spleen dilated again, respiration became slow and gasping, the leg volume remained reduced, and if the dose was large enough death occurred despite artificial respiration. These changes are illustrated in Fig. 2. When oxygenation was maintained by artificial respiration, the heart continued to act well and only failed 5–10 minutes after blood pressure had reached zero. The isolated perfused heart of the cat (Langendorff) showed no ill-effects from the injection of up to 5.0 mg. BAL and the rate of coronary flow was unchanged. The progressive fall in blood pressure with an active heart, in the presence of peripheral vasoconstriction, suggests a steady leakage of fluid from the circulation. The petechial haemorrhages seen on the lungs and liver indicate damage to capillaries and small vessels. The mean packed cell volume in five anaesthetized cats was 36.6 per cent (the blood having been spun for 25 min. at 5,000 r.p.m.); three hours after the administration of 100 mg. BAL/kg. and shortly before death took place it was 48.3 per cent, an increase of 32 per cent. This indicates a severe degree of haemoconcentration, despite the fact that an average of 25 ml. of saline and other solutions had been given intravenously in the course of the experiments, and it would appear to be the primary cause of death in anaesthetized animals, as Chenoweth (1946) indicated.

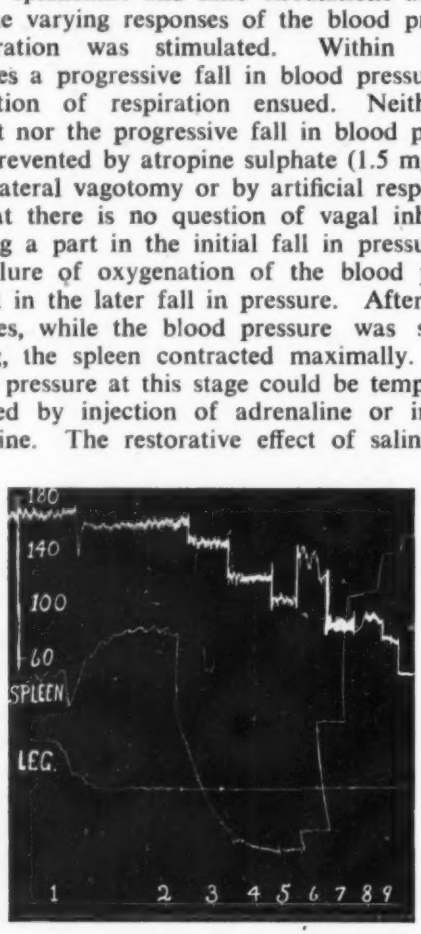

FIG. 2.—Cat, ♂ 3 kg. wt. Ether and chloralose, 80 mg./kg. Upper record carotid blood pressure, middle record splenic volume (plethysmograph and piston recorder), lower record leg volume (plethysmograph–tambour). Injection points and time in 30 secs. are marked. At 1, 40 mg.BAL/kg. was given i.v. at 11.43 a.m. and 20 mg./kg. repeated at 12.0 a.m., 1.56 p.m., and 2.10 p.m. (total 100 mg./kg.); 10.0 ml. saline at 12.21 temporarily restored the pressure. The time relationships are as follows: 1 = 11.43 a.m.; 2 = 11.50 a.m.; 3 = 11.54 a.m.; 4 = 12.06 p.m.; 5 = 12.24 p.m.; 6 = 1.45 p.m.; 7 = 1.53 p.m.; 8 = 2.10 p.m.; 9 = 2.20 p.m.

The odour of BAL could be detected in blood, urine, tears, and expired air, and in the freshly cut organs after death.

Action of BAL on the toxicity of metallic compounds. Arsenic

The arsenical preparation used was mapharside, freshly made up and injected in 0.25 ml. saline intraperitoneally into groups of white mice. The LD50 for mapharside alone was found to be 34.4 mg./kg.; BAL had a strongly protective action, raising the LD50 to 350 mg./kg. or tenfold. This action is illustrated in Fig. 3 and is in agreement with the findings of Stocken, Thompson, and Whittaker (1947) with rats.

Twenty-four guinea-pigs of 350–400 g. weight were given a daily injection of mapharside (15 mg./kg.) subcutaneously ; twelve were given in addition a daily injection of BAL (40 mg./kg.) intraperitoneally. Eight of the guinea-pigs receiving arsenic alone had died with symptoms of restlessness, twitching, diarrhoea, wasting, and weakness between the fifth and tenth day of injection, when the remainder were killed. The guinea-pigs receiving BAL remained well and were sacrificed on the tenth day. Fresh specimens of liver,

kidney, spleen and gut were sectioned, stained, and examined. The chief lesions caused by arsenic poisoning were necrosis of the liver lobules with much debris and exudate, severe disintegration of the glomeruli and tubules of the kidney, catarrhal changes in the mucous membrane of the gut and congestion of the spleen with necrotic changes in the pulp and nodules. These changes were prevented by BAL therapy, the most striking differences being in the kidney. It is evident that the combination of arsenic and BAL causes little tissue damage during its excretion.

Antimony

The preparation of antimony used was tartar emetic, which proved to have an LD50 of 56.2 mg./kg.; 40 mg. BAL /kg. i.p. had a protective action on mice poisoned with this compound, raising the LD50 to 71.6 mg./kg. The results are illustrated in Fig. 3.

Chromium

The preparation used was chromium trioxide, which had an LD50 of 66.3 mg./kg. BAL (40 mg./kg. i.p.) had a protective action against chromium poisoning, raising the LD50 to 85.8 mg./kg.; this effect is illustrated in Fig. 3.

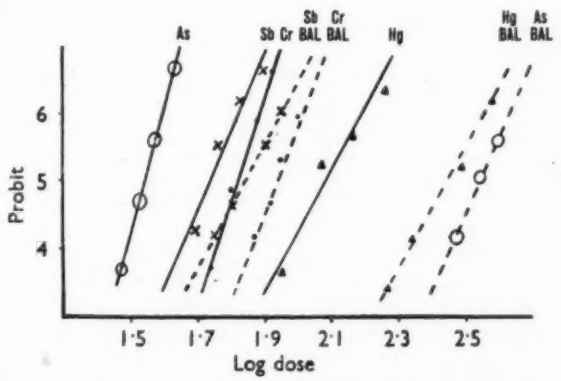

FIG. 3.—The protective action of BAL (40 mg./kg. i.p.) on the toxicity of arsenic (mapharside) antimony (tartar emetic), chromium (chromic acid) and mercury (corrosive sublimate) in groups of white mice. The ordinates are probits of lethality, the abscissae logs. of the dosage. BAL decreased the toxicity of each metal, an effect illustrated by the shift of the lines to the right.

Twelve guinea-pigs were shaved and given 0.25 ml. of 5 per cent (w/v) chromic acid solution intracutaneously as a wheal. After five days there were well-established circular ulcers at each site of injection with a black sloughing eschar about half an inch across and a raised red areola for a quarter-inch round the ulcer. The animals had recovered from the constitutional upset resulting from chromium absorption. At this point they were divided into two groups and half the animals treated with a daily application of acriflavine ointment to the ulcers; the other half received the same ointment made up to contain 10 per cent of BAL. The BAL ointment was kept cool between applications and retained its pungent odour to the end of the experiment. The ulcers treated with BAL became soft, filled in rapidly and had healed in 30 days; the ulcers treated with acriflavine alone were still indurated and approximately one quarter their original size after 40 days, when the animals were sacrificed. It is suggested that treatment with BAL ointment, and, if necessary, systematically administered BAL, would be of value in industrial chrome ulceration.

Mercury

The preparation tested was mercuric chloride, the LD50 of which was 120 mg./kg.; BAL had a protective effect on animals poisoned with this salt, raising the LD50 to 281 mg./kg. (see Fig. 3). According to Long and Farah (1946) intravenous injection of BAL reduces the lethality of intravenous 'salyrgan' in mice, and protects the cardiovascular system of anaesthetized dogs from the toxic effects of this mercurial compound. In the present work the LD50 of mersalyl B.P. solution was found to be 169 mg./kg. i.p. in mice. BAL (40 mg./kg. i.p.) immediately after the diuretic lowered the LD50 to 100 mg./kg. For mersalyl given intravenously the LD50 was 120 mg./kg. and BAL (20 mg./kg.) also given intravenously protected many of the mice from the violent convulsions caused by intravenous mersalyl and raised the LD50 to 165 mg./kg.

The effects of BAL are discussed later, but some clarification was provided by the results of tests of the effect of mersalyl on water diuresis in groups of rats. BAL (40 mg./kg. i.p.) acted as an antidiuretic, suppressing urine for 3–4 hours. Mersalyl (100 mg./kg. i.p.) caused immediate anuria and death followed after 48 hours, but if the two were given intraperitoneally within a few minutes of one another suppression was much less than with either alone, and the rats survived. In a double experiment using 16 rats the animals when watered and given subcutaneous saline had a diuresis of which the peak occurred after 65 min.; after subcutaneous mersalyl (1.0 mg./kg.) and saline the peak occurred at 72 min. Repetition of mersalyl (1.0 mg./kg.) and saline after one week gave a figure of 73 min., whereas mersalyl s.c. and BAL (4.0 mg./kg.) i.p. gave a figure of 90 min. It follows that mersalyl and BAL in large doses each have a delaying effect on the excretion of urine from normal rats and that given together they tend to cancel one another; with small doses this effect is not seen.

Chronic mercurial poisoning was induced in 12 rabbits by giving them mercury perchloride (1.0 mg./kg.) for ten days, and in 16 guinea-pigs by giving

5.0 mg./kg. i.p. daily. The animals so treated lost weight and developed diarrhoea and tremors and died, the guinea-pigs living for only 4 days, the rabbits for 12–16 days. Half the rabbits were also given 2.5 mg. BAL/kg. s.c. daily, and half the guinea-pigs 40 mg./kg. The animals given BAL survived in good health until sacrificed. Microscopy revealed marked necrosis of liver, kidney, and gut in the animals poisoned with mercury, and an absence of these lesions in the animals protected with BAL.

Lead

The salt used was plumbi acetatis B.P., the LD50 of which was 461 mg./kg. i.p.; BAL (40 mg./kg. i.p.) had an additive effect on the toxicity of this substance, the LD50 falling to 416 mg./kg.; this effect is shown in Fig. 4.

Gold

The salt used was sodium aurothiomalate and the LD50 found was 1.096 g./kg. i.p.; BAL had an additive effect on the toxicity of this compound, reducing the LD50 to 812 mg./kg., an effect shown in Fig. 4. Successful clinical use of BAL in cases of gold poisoning is reported by Cohen, Goldman, and Dubbs (1947).

Bismuth

The salt tested was sodium and potassium bismuth tartrate, the LD50 of which was found to be 676 mg./kg. i.p. BAL had an additive effect on the toxicity of this compound, reducing the LD50 to 288 mg./kg.; this effect is shown in Fig. 4.

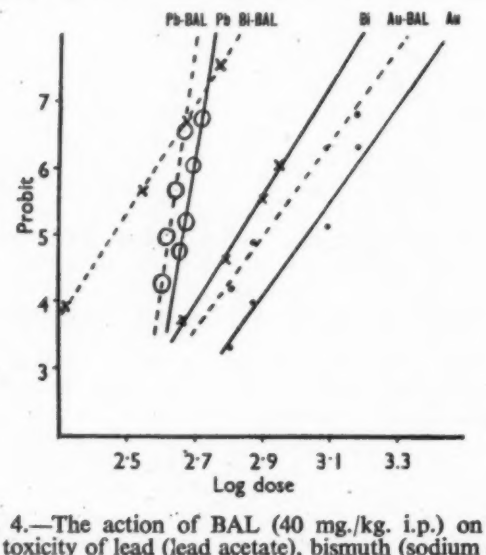

FIG. 4.—The action of BAL (40 mg./kg. i.p.) on the toxicity of lead (lead acetate), bismuth (sodium and potassium bismuth tartrate) and gold (sodium auro-thiomalate) in groups of white mice. The ordinates are probits of lethality and the abscissae logs. of dosage. BAL increased the toxicity of each metal, an effect illustrated by the shift of the lines to the left.

Effect of BAL on the action of insulin

Barron, Miller, and Meyer (1947) state that the action of insulin (0.8 U./kg.) is inhibited by BAL in doses of 0.1 g./kg. i.v. Larger doses of BAL reduce the blood sugar of rabbits and prove fatal.

In the present experiments a group of five rabbits about 2.0 kg. weight were starved overnight and a blood sample of 0.5 ml. drawn from the marginal ear vein of each rabbit and pooled in a heparinized tube. The animals were given 0.5 U./kg. soluble insulin subcutaneously and pooled blood samples collected every hour. After three days the experiment was repeated with the addition of 25 mg. BAL/kg. i.p. The mean blood sugar level after three hours had fallen by 75 per cent with the animals given insulin alone, whereas with insulin and BAL the level at three hours had fallen by 21 per cent. The insulin by itself gave a more precipitate and a more prolonged fall in blood sugar level than did the insulin in the same animals treated with BAL. This inhibitor effect of BAL on the action of insulin is illustrated in Fig. 5.

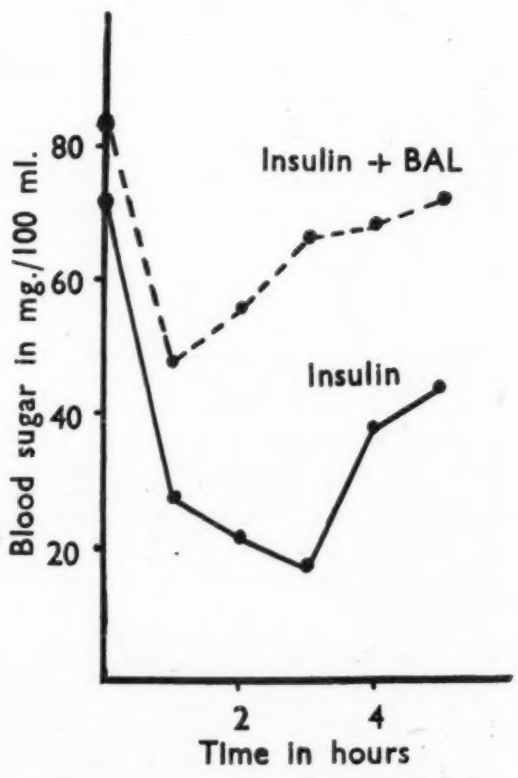

FIG. 5.—The mean blood sugar curve in five rabbits after 0.5 U./kg. soluble insulin s.c., and the effect of BAL (25 mg./kg. i.p.) in reducing the effect of the same dose of insulin in the same animals.

Discussion

It has been shown in the present work and in the work of others quoted that BAL is an effective antidote to poisoning by arsenic, mercury, antimony, chromium, nickel, and cadmium. McCance and Widdowson (1946) found that the closely related BAL-glucoside promotes excretion of copper and zinc salts, and it has further been shown that iron and thallium are unaffected by thiol compounds whereas lead and selenium are rendered more toxic. Bismuth and gold were found to be made more toxic to mice by BAL in the present work but according to Braun *et al.* (1946) BAL rendered bismuth less toxic to rabbits and according to Ragan and Boots (1947) gold less toxic to man. These differing results together with the apparently contradictory results obtained in the present work with mersalyl, and also the complex manifestations of the toxicity of BAL itself are attributable to the presence of sulphydryl groups in its molecule. Webb and van Heyningen (1947) point out that BAL inhibits the activity of any enzyme which contains a heavy metal, capable of being linked with available SH, as prosthetic group to the protein moiety of the enzyme. Such an activity would account for the widely differing phenomena of BAL poisoning—convulsive action on the C.N.S., drop in blood sugar, interference with respiration, and presumably also the damage to the small vessels which produces such marked alterations in the circulation. The smooth muscle of peripheral vessels appears to be much more sensitive to BAL than the muscle of gut, uterus, the heart, or coronary arteries, and the differing initial response of the spleen and limb volumes to intravenous injection of BAL needs further examination.

Gilman *et al.* (1946) point out that with cadmium the compound formed *in vivo* by BAL is a soluble substance which proves on isolation to have a greater toxicity than cadmium chloride itself. This increased toxicity is caused by increased pathogenicity to the kidney. The arsenic, mercury, chromium, and antimony preparations used to determine the LD50 in mice are highly irritant to the peritoneum, quickly absorbed and cause gross renal damage on excretion; intraperitoneal treatment with BAL reduced the toxicity of these compounds and prevented visible damage to the kidney. The products of linkages between BAL and these metals must be of such a nature that they are not toxic to the kidney on excretion. The reduction of acute toxicity of the metallic compound may be due to production of a non-irritant compound by reaction with BAL in the peritoneal cavity, and this compound may be absorbed more slowly and excreted in a non-toxic form. The salts of gold, bismuth, and lead which were used have a much higher LD50 than the group of metals discussed above, when given intraperitoneally. They are not so irritant as the former; they tend to be precipitated and to be absorbed slowly, and thus the cause of their acute toxicity is probably different from that of the others. BAL-metal complexes with these metals may be absorbed more quickly or be more toxic than the metallic salts themselves.

A point of importance is suggested by the finding of Gilman *et al.* (1946) that the BAL-cadmium complex formed *in vitro* differs markedly from the complex formed *in vivo*. In the present work with mice the metallic salts were given intraperitoneally followed by the BAL at the same site. If an *in vitro* type of precipitate were formed in the peritoneal cavity, this might well differ in stability, absorption rate, and toxicity from the type of compound formed *in vivo* in rabbits which were injected intramuscularly at different sites with bismuth and BAL by Braun *et al.* (1946) or from that in man given gold and BAL at separate sites and times by Ragan and Boots (1947). The same phenomenon of formation of different mercaptides with different toxicities according to the conditions of reaction of the metal and the thiol compound may account for the opposite effects of BAL on the toxicity of mersalyl given intraperitoneally and intravenously.

The many toxic properties of BAL and the variability of its effects indicate that some other related compound would be preferable for therapeutic use. Danielli, Danielli, Mitchell, Owen, and Shaw (1946) give evidence that BAL-glucoside may be suitable.

Summary

British Anti-Lewisite (BAL) has an LD50 of 100 mg./kg. i.p. in white mice. It causes conjunctivitis, ataxia, rapid and then impaired respiration and convulsions in small mammals. In anaesthetized rabbits small doses (4 mg./kg.) cause a temporary rise in blood pressure, but in cats only a fall in pressure is seen; this is considered to be due initially to splanchnic dilatation, but the main effect on the circulation of anaesthetized cats is a progressive fall in blood pressure despite constriction of the leg and spleen and an active heart. Loss of fluid from the capillaries leading to haemo-concentration and a state of shock is held to be the cause of death in anaes-

thetized animals, whereas convulsion is the cause of death in intact animals.

BAL has a protective action on white mice poisoned with arsenic, mercury, antimony, and chromium and a deleterious effect on mice poisoned with lead, gold, and bismuth. It prevents or relieves tissue damage caused by chronic poisoning with arsenic, mercury and chromium. BAL inhibits the action of insulin. Some apparent anomalies in the detoxifying action of BAL are discussed.

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